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PREVALENCE OF DENTINOGENESIS IMPERFECTA AND DENTAL ABERRATIONS RELATED TO GENETIC FINDINGS IN OSTEOGENESIS IMPERFECTA

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Prevalence of dentinogenesis imperfecta and dental aberrations related to genetic findings in osteogenesis imperfecta

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To all individuals with osteogenesis imperfecta and dentinogenesis imperfecta

ABSTRACT

Osteogenesis imperfecta (OI) is a clinically and genetically heterogeneous connective tissue disorder, mainly caused by mutations in *COL1A1* and *COL1A2*, the genes encoding collagen type I. The cardinal symptoms include bone fragility and varying degrees of growth retardation. Dental and craniofacial manifestations, with dentinogenesis imperfecta (DGI) being the major one, are common findings in OI. DGI exists in two forms, DGI type I – a collagenous manifestation associated with OI, and DGI type II – a non-collagenous disease caused by mutations in the *DSPP* gene. Clinically and radiographically the discrimination between the two is challenging.

Study I of this thesis had two aims, to: *(i)* investigate the prevalence and incidence of DGI type II among Swedish children and adolescents and *(ii)* identify undiagnosed cases of DGI type I. We invited all specialist pediatric dental clinics (n=47) in Sweden to participate in the study. Pediatric dentists interviewed and examined patients regarding medical and dental aspects known to be associated with OI. The prevalence of DGI type II was estimated to be 0.0022% (95% CI: 0.0016–0.0029%) or 2.2 in 100,000 individuals. Clinical and radiographic findings raised a suspicion of undiagnosed OI in one individual, who was later diagnosed with OI type IV.

Study II was a cohort study comprising 128 unrelated individuals with OI. Panoramic radiographs were analyzed regarding DGI, and congenitally missing teeth. Collagen I genes were Sanger sequenced in all individuals. Tooth agenesis was diagnosed in 17% (hypodontia 11%, oligodontia 6%) and was more frequent in those with DGI (p=0.016) and in those with OI type III, 47%, compared to those with OI types I, 12% (p=0.003) and IV, 13% (p=0.017). Seventy-five percent of the individuals with oligodontia harbored a mutation predicted to cause a qualitatively changed protein, but there was no association with type of OI, gender, or presence of DGI.

Study III investigated the association between collagen I mutations and DGI, taurodontism, and retention of permanent second molars in a retrospective cohort of 152 unrelated children and adolescents with OI. DGI was diagnosed clinically and radiographically in 29% of the individuals (44/152) and through isolated histological findings in another 19% (29/152). In the individuals with a *COL1A1* mutation, 70% (7/10) of those with a glycine substitution located C-terminal of p.Gly305 exhibited DGI in both dentitions while no individual (0/7) with a mutation N-terminal of this point exhibited DGI in either dentition (p=0.01). In the individuals with a *COL1A2* mutation, 80% (8/10) of those with a glycine substitution located C-terminal

of p.Gly211 exhibited DGI in both dentitions while no individual (0/5) with a mutation N-terminal of this point (p=0.007) exhibited DGI in either dentition. DGI was restricted to the deciduous dentition in 20 individuals. Taurodontism occurred in 18% of the lower permanent first molars and retention of permanent second molars in 31% of the adolescents.

Study IV comprised 11 individuals with OI and hypodontia/oligodontia who were clinically examined and further genetically investigated with whole-genome sequencing. We detected a novel homozygous nonsense variant in *CREB3L1,* p.Tyr428*, c.1284C>A in one boy previously diagnosed with OI type III. *COL1A1* and *COL1A2* were the only two genes with variants that could be detected in all individuals. However, we found rare variants of unknown significance in several other genes related to tooth development. Among those, a missense variant in *AXIN2*, segregating with tooth agenesis in an autosomal dominant manner in the family of a boy with OI type IV and oligodontia.

Conclusions This thesis found that the prevalence of DGI type II in Sweden was significantly lower than previously reported, and points to the importance of excluding OI in children with DGI. Tooth agenesis is common in OI, and OI caused by a collagen I mutation predicted to induce a qualitatively changed protein is associated with oligodontia. Additive effects from other modifying or interacting genes may contribute to the severity of the expressed phenotype. Dental aberrations are more common in individuals with a qualitatively changed collagen type I. The varying expressivity of DGI is related to the location of the collagen I mutation.

LIST OF SCIENTIFIC PAPERS

I. **Andersson K**, Malmgren B, Åström E, Dahllöf G. Dentinogenesis imperfecta type II in Swedish children and adolescents. Submitted.

II. Malmgren B, **Andersson K**, Lindahl K, Kindmark A, Grigelioniene G, Zachariadis V, Dahllöf G, Åström E. Tooth agenesis in osteogenesis imperfecta related to mutations in the collagen type I genes. Oral Dis. 2017 Jan;23(1):42-49.

III. **Andersson K**, Dahllöf G, Lindahl K, Kindmark A, Grigelioniene G, Åström E, Malmgren B. Mutations in *COL1A1* and *COL1A2* and dental aberrations in children and adolescents with osteogenesis imperfecta - A retrospective cohort study. PLoS One. 2017 May 12;12(5):e0176466.

IV. **Andersson K**, Malmgren B, Åström E, Nordgren A, Taylan F, Dahllöf G. Mutations in *COL1A1/A2* and *CREB3L1* are associated with oligodontia in osteogenesis imperfecta. In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

BMP	Bone morphogenetic protein
CADD	Combined annotation dependent depletion
Collagen I	Collagen type I
COL1A1	Collagen type I gene encoding the proα1(1) chain
COL1A2	Collagen type I gene encoding the proα2 (1) chain
C-terminal	Carboxy-terminal
DD	Dentin dysplasia
DGI	Dentinogenesis imperfecta
DGI-I	Dentinogenesis imperfecta type I
DGI-II	Dentinogenesis imperfecta type II
DSPP	Dentin sialophosphoprotein
DPP	Dentin phosphoprotein
ECM	Extracellular matrix
GERP	Genomic evolutionary rate profiling
HERS	Hertwig's epithelial root sheath
MLPA	Multiplex ligation-dependent probe amplification
N-terminal	Amino-terminal
OI	Osteogenesis imperfecta
OMIM	Online Mendelian inheritance in man
PTC	Premature termination codon
MIM	Mendelian Inheritance in Man
SNV	Single nucleotide variant
SIBLINGs	Small integrin-binding ligand, N-linked glycoproteins
TGFβ	Transforming growth factor beta
WGS	Whole genome sequencing

INTRODUCTION

Osteogenesis imperfecta (OI) is a heterogeneous heritable connective tissue disorder. Mutations in the genes encoding collagen type I, *COL1A1* and *COL1A2*, are the main cause of these disorders. Cardinal symptoms include bone fragility and varying degrees of growth retardation. The spectrum or oral manifestations and their genetic causes are currently largely unknown. Among the oral manifestations of OI, dentinogenesis imperfecta (DGI) is the most common. Bone and dentin share many characteristics and have a similar composition. Collagen type I is the main organic component of both tissues, which is also the main organic component of the craniofacial bone and is thus important in craniofacial development. DGI exists in two forms: DGI type I – a collagenous manifestation associated with OI, and DGI type II – a non-collagenous disease caused by mutations in the *DSPP* gene. Clinically and radiographically, discriminating between the two is challenging. Prevalence of DGI type II is unknown in Sweden and other countries.

The start of this project can be traced to 1991 when the Swedish national multidisciplinary pediatric OI team at Astrid Lindgren Children's Hospital at Karolinska University Hospital in Stockholm was formed. Dr. Barbro Malmgren was the first pediatric dentist on the team and made great efforts to document the clinical, radiographic, and histologic findings of the patients. To deepen our understanding of genotype-phenotype correlations in children and adolescents with OI using DNA analysis, a research collaboration was established with Uppsala University in 2006.

This thesis assesses the frequency of DGI; it also investigates the spectrum of oral manifestations in OI and relates them to genetic findings for the purpose of broadening our understanding of the variability in disorder expression.

Osteogenesis imperfecta

OI is a clinically and genetically heterogeneous connective tissue disorder characterized primarily by an increased tendency for fractures throughout life. Other symptoms include growth deficiency, joint laxity, tendency toward prolonged bleeding, bruises, premature hearing loss, blue sclerae, and DGI. The reported prevalence of OI is approximately 7 in 100,000 individuals (Lindahl et al., 2015). Severity ranges from a slight increase in fracture tendency to perinatal lethality (Sillence et al., 1979). Sillence categorized patients with OI into four main types: (I) dominantly inherited OI with blue sclerae, (II) lethal perinatal OI with

radiographically crumpled femora and beaded ribs, (III) progressively deforming OI, and (IV) dominantly inherited OI with normal sclerae. The type numbers, I–IV, were based on the order in which the syndromes first appeared in the manuscript. A subsequent categorization was later performed of OI type I and IV based on absence (A) or presence (B) of DGI (Table 1).

Table 1. Classification of OI modified from Sillence et al., (1979).

OI type	DGI*	Clinical features	Inheritance**
IA	-	Normal or short stature Little or no bone deformity Blue sclerae Hearing loss common	AD
IB	+		
II	?	Severe osseous fragility, perinatally lethal	AD (*de novo* mutations) AR (rare)
III	+/-	Very short stature Progressively deforming bones Scleral hue varies Hearing loss less common than type I	AD AR (uncommon)
IVA	-	Variable short stature Mild to moderate bone deformity Normal sclerae Hearing loss less common than type I	AD
IVB	+		

*DGI = dentinogenesis imperfecta; **AD = autosomal dominant;
AR = autosomal recessive

The etiology of OI is either directly or indirectly related to disturbances in collagen type I, the main organic component of bone and dentin. Approximately 95% of all collagen content of bone and about 80% of total bone protein is collagen type I (Viguet-Carrin et al., 2006). The collagen type I molecule is composed of three α chains: two α1 chains and one α2 chain. These chains are synthesized as procollagen from *COL1A1* (17q21.33) and *COL1A2* (7q21.3), located on chromosomes 17 and 7, respectively. The first genetic cause of OI to be described, an internal deletion located in *COL1A1*, was observed in an individual with OI type II in 1983 (Chu et al., 1983). The allelic heterogeneity seen in OI was elucidated in following years. Variants in *COL1A1* and *COL1A2*, the genes encoding collagen type I, were detected in the majority of affected individuals.

In some families, however, the genetic cause was unknown. The first recessive variant responsible for OI type II was identified in the *CRTAP* gene in 2006 (Barnes et al., 2006). Autosomal dominant mutations in *COL1A1* or *COL1A2* have traditionally been thought to

cause over 90% of OI cases (van Dijk et al., 2012, Pepin & Byers, 2015). However, more recent studies indicate a lower prevalence (Bardai et al., 2016). Dominant mutations in *IFITM5* and *WNT1* may also cause OI (Semler et al., 2012, Cho et al., 2012, Laine et al., 2013). In other cases, pathogenic variants in non-collagenous genes are the cause of recessive forms of the disorder. An X-linked recessive mutation has also been identified. To date, mutations in 17 genes have been associated with OI. The recessive variants are found in genes that are involved in collagen biosynthesis, processing, and posttranslational modifications (Kang et al., 2017).

This new knowledge of the genetic heterogeneity stressed the need for an updated, complementary grouping; the original Sillence criteria were mainly based on clinical and radiographic findings. At the 2009 meeting of the International Nomenclature group for Constitutional Disorders of the Skeleton, the original four groups were expanded by a fifth, OI type V (Van Dijk & Sillence, 2014). The classification is phenotypic, and the groups are genotypically heterogeneous (Table 2).

Collagen defects in osteogenesis imperfecta

Individuals of European descent with OI type I–IV are heterozygous for mutations in the *COL1A1* and *COL1A2* genes in most cases. Sibling recurrences without an affected parent may be due to gonadal mosaicism for heterozygous dominant mutations (Van Dijk & Sillence, 2014). Autosomal recessive variants are more common in cases of consanguinity. Two main classes of mutations in collagen type I result in OI – those causing a quantitatively changed protein and those inducing qualitative changes in collagen type I. Quantitative defects are characterized by about a 50% reduction of normal type I procollagen secretion; qualitative defects are characterized by secretion of structurally abnormal type I procollagen molecules (Wenstrup et al., 1990). The first class of mutations usually produces premature termination codons (PTC) in the coding sequence of one *COL1A1* allele (Marini et al., 2007). These PTCs cause nonsense mediated decay of the mRNA derived from that allele and a premature termination of translation (Willing et al., 1992, Willing et al., 1996, Redford-Badwal et al., 1996). This results in matrix insufficiency and the phenotype of OI type I.

Table 2. New osteogenesis imperfecta (OI) nomenclature. Modified from (Van Dijk & Sillence, 2014).

Type	Gene	MIM #	Locus	Protein product	Inheritance
Non-deforming OI, blue sclerae					
1	*COL1A1*	166200	17q21.33	Collagen α1(I) chain	AD
	COL1A2	166200	7q22.3	Collagen α2(I) chain	AD
Common variable OI, normal sclerae					
4	*COL1A1*	166200	17q21.33	Collagen α1(I) chain	AD
	COL1A2	166200	7q22.3	Collagen α2(I) chain	AD
	WNT1	615220	12q13.12	Wingless-type mouse mammary tumor virus (MMTV), integration site family, member 1	
	CRTAP	610682	3p22.3	Cartilage-associated protein (CRTAP)	AR
	PPIB	259440	15q22.31	Cyclophilin B (CyPB)	AR
	SP7	613849	12q13.13	Osterix	AR
	PLS3	300910	Xq23	Plastin 3	XL
OI, calcification in interosseous membranes					
5	*IFITM5*	610967	11p15.5	Interferon-induced transmembrane protein 5	AD
Progressively deforming					
3	*COL1A1*	166200	17q21.33	Collagen α1(I) chain	AD
	COL1A2	166200	7q22.3	Collagen α2(I) chain	AD
	BMP1	614856	8p21.3	Bone morphogenetic protein 1	AR
	CRTAP	610682	3p22.3	Cartilage-associated protein (CRTAP)	AR
	FKBP10	610968	17q21.2	Peptidyl-prolyl cis-transisomerase (FKBP10)	AR
	LEPRE1	610915	1p34.2	Prolyl 3-hydroxylase 1 (P3H1)	AR
	PLOD2	609220	3q24	Procollagen-lysine, 2-oxoglutarate, 5-dioxygenase 2	AR
	PPIB	259440	15q22.31	Cyclophilin B (CyPB)	AR
	SERPINF1	613982	17p13.3	Pigment-epithelium-derived factor (PEDF)	AR
	SERPINH1	613848	11q13.5	Heat shock protein 47 (HSP47)	AR
	TMEM38B	615066	9q31.1	Trimeric intracellular cation channel B (TRIC-B)	AR
	WNT1	615220	12q13.12	Wingless-type mouse mammary tumor virus (MMTV) integration site family, member 1	AR
	CREB3L1	616229	11p11.2	Old astrocyte specifically induced substance (OASIS)	AR
Perinatally lethal					
2	*COL1A1*	166200	17q21.33	Collagen α1(I) chain	AD
	COL1A2	166200	7q22.3	Collagen α2(I) chain	AD
	CRTAP	610682	3p22.3	Cartilage-associated protein (CRTAP)	AR
	LEPRE1	610915	1p34.2	Prolyl 3-hydroxylase 1 (P3H1)	AR
	PPIB	259440	15q22.31	Cyclophilin B (CyPB)	AR

AD, Autosomal dominant; AR, Autosomal recessive; XL, X-linked

Qualitative defects are often caused by substitutions for one of the glycine residues in the triple helical domain in one of the α1 or α2 chains (Kuivaniemi et al., 1997, Forlino & Marini, 2000). The mutations cause broad expressivity of OI. In the coding sequence of the triple helical domain, which can be written $GGNNNNNNN_{338}$, substitutions at either G will cause a substitution for glycine. Substitutions at two out of every nine nucleotides will result in a clinically apparent phenotype (Marini et al., 2007). Based on current knowledge of genotype-phenotype correlations, this factor should not be the only deciding variable in pregnancy termination.

Alterations in splice sites are common in OI (Kuivaniemi et al., 1997). These changes can cause intronic inclusion, exon skipping or activation of cryptic splice sites in introns or exons. The effects on mRNA and protein depend on whether the alterations are in-frame or produce translational frameshifts. Qualitative defects have more deleterious consequences for the extracellular matrix (ECM) than quantitative defects (Forlino & Marini, 2000). A small group of insertions, deletions, and duplications in the collagen helix as well as alterations in the C-terminal propeptide may also have a resulting OI phenotype.

Diagnostics of OI

A thorough medical and family history, a physical examination, a radiographic evaluation, and laboratory testing are often needed in order to set a positive diagnosis of OI (Byers et al., 2006). The timing of the diagnosis and extension of the investigation depend on several factors. More severe cases of OI (types II, III, and severe type IV) may often be detected by fetal ultrasound examination and confirmed by genetic analysis. In childhood, typical radiographic findings of moderate to severe OI include bowing of the humerus and femora, and wormian bones in cranial sutures. Fractures, vertebral compression, and osteopenia frequently occur. The diagnosis of milder forms may be more challenging. In these cases, presence of DGI, blue sclerae, short stature, and joint hypermobility may be important findings. Bone mineral density can be assessed by dual-energy X-ray absorptiometry. Finally, DNA-based tests using blood or saliva as a source can be important tools for confirming a diagnosis.

Treatment of children with OI

Due to the wide range of severity, individualization of treatment is important. A combination of treatment modalities is often needed and leading OI-centers use a multidisciplinary team approach. New insights into the field of muscle-bone interactions (crosstalk) support the importance of loading of the skeleton preferably by standing and walking. Medical treatment of OI with intravenous bisphosphonates has been used since 1991 in individuals with severe

forms of OI (Astrom & Soderhall, 1998, Glorieux et al., 1998, Rauch et al., 2003). Bisphosphonates inhibit osteoclasts and bone resorption which can increase bone mass and density (Rauch et al., 2003). When indicated, treatment onset can be early in infancy. Physical and occupational therapies are important for improving activities of daily life and motor function and for exercise of the cardiovascular system. Osteotomies and insertion of intramedullary rods are often needed in more severe forms of OI for stabilization and correction of long bone deformities. Rodding can also be used in milder cases with repeated long bone fractures.

Collagen biosynthesis

Collagen type I is the major organic component of bone and dentin, and the protein product that is affected in OI and DGI type I. The synthesis of collagen type I is a complex process involving several crucial steps. Collagen type I is a heterotrimer composed of two α1 chains and one α2 chain that are wrapped around each other in a rope-like manner, thus forming a triple helix (Fig. 1). The α chains are encoded by two genes, *COL1A1* and *COL1A2*, located on chromosomes 17 and 7, respectively. Both genes contain more than 50 exons coding for the protein sequence. The major part of the protein is composed of the triple helical domain comprising 1014 amino acids that are encoded by 43 exons. The region encodes sequences of repeating triplets of the tripeptide Gly-Xaa-Yaa, where Gly is glycine and X and Y are often proline and lysine residues. The remaining exons are located in the amino (N)-terminal and carboxy (C)-terminal domains of the genes. These exons encode the signal sequence and proteolytic cleavage sites (N-terminal region), and a proteolytic cleavage site and a globular domain of importance for molecular assembly in the C-terminal portion of the gene (Byers et al., 1991).

The mRNA transcripts of *COL1A1* and *COL1A2* are translated in the rough endoplasmic reticulum. The α chains subsequently undergo posttranslational modification including hydroxylation and glycosylation of proline and lysine residues by a number of hydroxylases. Once the C-terminal propeptides of the chains are synthesized, intra-chain disulfide bonds are formed. The propeptides are essential for the correct selection and 2:1 proportion of the α chains, and the triple helix nucleus is stabilized by the disulfide bonds (Marini et al., 2017).

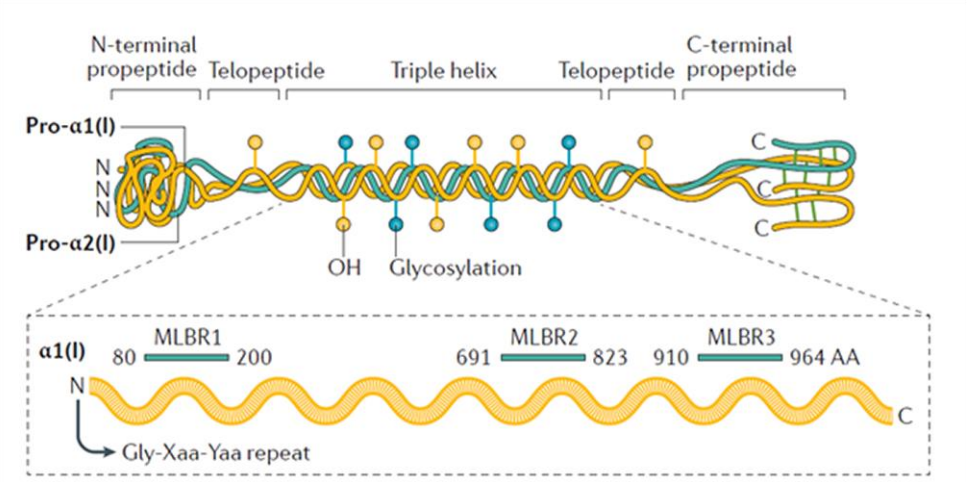

Figure 1. Collagen type I structure. The molecule is a heterotrimer composed of two α1 chains and one α2 chain. N- and C-terminal propeptides are retained during secretion of the procollagen molecules but are proteolytically cleaved after secretion. Glycine is mandatory at every third position and is flanked by other amino acids, most often proline and hydroxyproline (X and Y). Posttranslational modification of proline is characterized by hydroxylation and glycosylation that is necessary for the stability of the triple helix. Major ligand-binding regions (MLBR) are regions important for the interaction of collagen type I with other collagen-related molecules and the ECM. Adapted from Marini et al. (2017).

Hydroxylation and glycosylation of the chains continue until a stable helix structure is formed. When this happens, the helix is no longer a substrate (Byers et al., 1991). It is essential for the integrity of the molecule that there is a glycine in every third position. The reason is that this is the only amino acid that sterically fits into the center of the triple helix. N- and C-terminal propeptides flank the central helical portion of the molecule (Marini & Blissett, 2013). Once the procollagen chains are assembled and the posttranslational modification of lysyl and prolyl residues is completed in the endoplasmic reticulum, procollagen molecules are transported to the Golgi apparatus. The procollagen molecules are packed into vacuoles and transported to the cell membrane. From here, the molecules are secreted into the pericellular space, and the mature collagen molecule is formed by proteolytic cleavage of the N- and C-terminal ends (Kuznetsova & Leikin, 1999). The monomers finally self-assemble into heterotrimeric collagen fibrils in the ECM. The fibrils are stabilized by inter- and intra-chain covalent crosslinking between specific domains. The formation and organization of fibrils are dependent on normal collagen type I molecules, and incorporation of abnormal molecules may have deleterious effects on the tissue.

To put the findings of this thesis in their correct context, fundamental knowledge of several tissues and developmental processes are necessary. These areas include bone formation and

structure, craniofacial development, tooth development, and dentin formation and composition in health and disease.

Bone

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ makes up the majority of the mineral phase, approximately 70–90%. The organic phase is composed of approximately 90% collagen type I, 5% non-collagenous proteins, and 2% lipids by weight (Young, 2003). Collagen type I acts like reinforcing bars in concrete and is essential for skeletal resistance to bending and torsion. The high amount of hydroxyapatite crystals in bone provide mechanical rigidity and the strength to withstand compression forces (Wang et al., 2001). Humans have two main types of bone: cortical (80%) and cancellous/trabecular (20%) bone. Cortical bone is solid, making up the outermost part of the bones, while cancellous bone is more porous and harbors the bone marrow. Several cell types are relevant to bone tissue, including osteoblasts, osteoclasts, bone lining cells, and osteocytes. Bone is an active tissue that is constantly remodeled during life. This process is orchestrated by the bone remodeling unit consisting of resorptive osteoclasts, osteoid-secreting osteoblasts, and osteocytes (terminally differentiated osteoblasts embedded in the bone matrix).

Alveolar bone

The alveolar bone, along with the periodontal ligament (PDL) and cementum, are the tissues that support the teeth. Alveolar bone, which forms the maxilla and the mandible, arises through intramembranous bone formation (Kruijt Spanjer et al., 2017). This differs from the embryogenesis of long bones, which requires endochondral bone formation (Mackie et al., 2008). Once formed, alveolar bone is composed of cortical bone separated by cancellous bone; the concentration of collagen type I is high. Collagen type I is organized in parallel fibrils that form the lamellae. The cortical bone of the alveolar processes is similar to bone in other parts of the skeleton. It contains Haversian systems with blood vessels and nerves that are important for bone remodeling and maintenance. The cancellous bone harbors hematopoietic cells, osteogenic cells, and adipose tissue.

Tooth development

Teeth are the result of an intricate system of morphogenetic tissue interactions in the embryo and child. These interactions are strictly regulated, sequential, and reciprocal; they are mediated by several transcription factors and conserved families of signaling molecules including Wingless-type MMTV integration site family members (Wnts), sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) (Balic & Thesleff,

2015). Tooth development is characterized by reciprocal interaction between epithelial and mesenchymal tissue (Thesleff, 2006). Morphologically and molecularly, early tooth development resembles the development of other organ systems (Fig. 2).

This regulatory communication between tissues is essential; it regulates the initiation and morphogenesis of teeth and affects differentiation of ameloblasts and odontoblasts, the cells responsible for secretion of the enamel and dentin matrices (Thesleff, 2003, Thesleff & Nieminen, 1996, Thesleff et al., 2007, Balic & Thesleff, 2015).

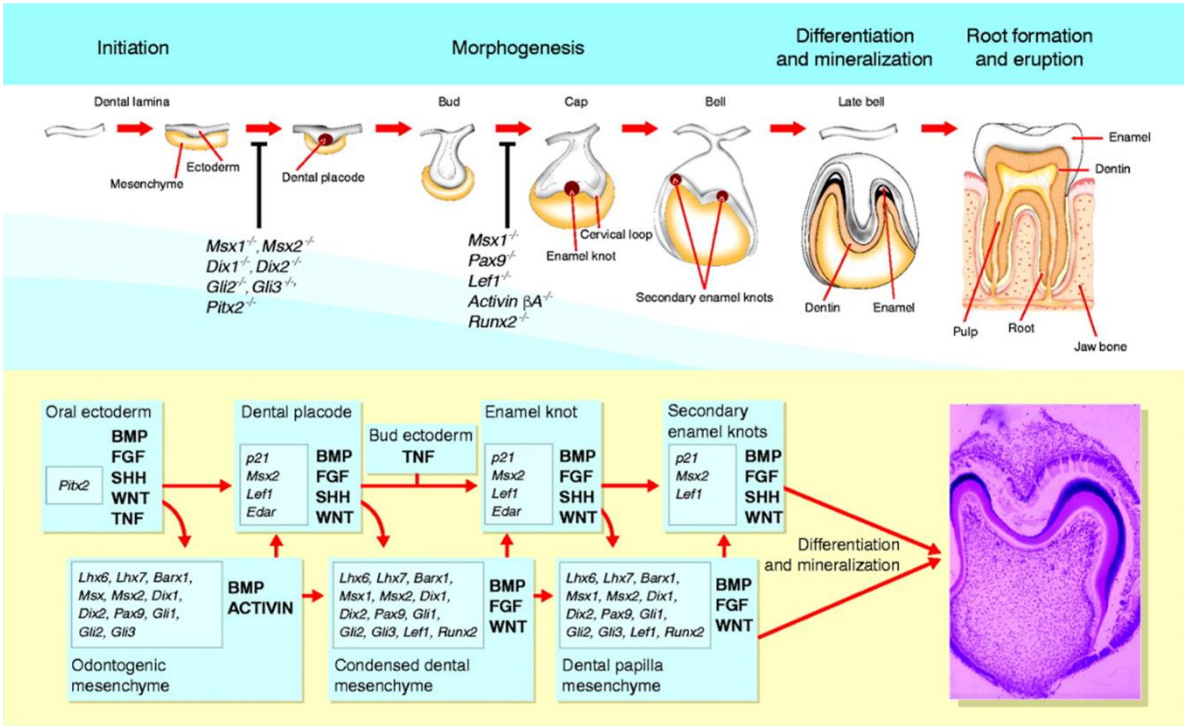

Figure 2. Schematic summary of tooth development. Sequential reciprocal interaction between the epithelial and mesenchymal tissues governs the morphogenesis of the tooth organ. Dentinogenesis starts during the advanced bell stage. After a layer of dentin has been formed, amelogenesis continues. All stages are dependent on the correct expression of several genes encoding signaling molecules and transcription factors. BMP, Bone morphogenetic protein; FGF, Fibroblast growth factor; SHH, Sonic hedgehog; WNT, Wingless-type MMTV integration site family; TNF, Tumor necrosis factor. Modified from (Thesleff, 2003).

In the initial stages of tooth development, the ectoderm buds into the neural-crest derived mesenchyme. Signals are sent from the ectoderm to the mesenchyme, which then condenses around the epithelial bud during the bud stage as epithelial signaling centers appear. Morphogenesis continues during the cap stage as the epithelium folds and grows to surround the dental papilla mesenchyme. Folding continues in the bell stage when the odontoblasts and

ameloblasts differentiate at the interface of the epithelium and mesenchyme. The secretion of dentin and enamel to form an ECM begins in this stage of tooth development (Thesleff, 2003).

The initial capacity for tooth formation resides within the epithelial tissue (Mina & Kollar, 1987). The epithelial signaling centers, primary enamel knots, express a significant number of signaling molecules. Tooth development starts by the expression of BMPs and FGFs that induce the expression of several transcription factors (Msx1, Msx2, Pax9, Dlx1, Dlx2, Lhx6, Lhx7, and Runx2) in the mesenchyme necessary for further odontogenesis. The induction of the mesenchyme results in a response to the oral epithelium to form the dental placode. Only the BMP and FGF families of signaling molecules perform a bidirectional signaling between the mesenchymal and epithelial tissues. One example is the expression of the transcription factor *Runx2*, mediating the expression of mesenchymal Fgf3 as a response to epithelial expression of Fgf4 (Aberg et al., 2004).

The Wnt/β-catenin signaling is important for transition from dental lamina to the placode stage. Inhibition of Wnt signaling by overexpression of Dkk1 arrests tooth development at the dental lamina stage (Liu et al., 2008). Overexpression of β-catenin drives continuing formation of ectopic signaling centers (Jarvinen et al., 2006). The transition from bud to cap stage is regulated by mesenchymal expression of *BMP4* resulting in formation of the enamel knot by induction of *p21*, causing the knot cells to exit the cell cycle (Jernvall et al., 1998).

Eda, a TNF signaling molecule, regulates development of ectodermal appendages. It has been shown that Eda plays important roles in intraepithelial signaling and in regulation of the signaling centers (Laurikkala et al., 2001, Haara et al., 2012). Wnt signaling governs Eda expression. Edar, the Eda receptor, is activated in the enamel knot in response to Eda. The Eda-Edar interaction regulates the formation and signaling activity of the enamel knots. The enamel knots regulate further tooth morphogenesis by producing signaling molecules and inhibitors, thus inducing responses in the epithelial and mesenchymal tissue. Shh is the only member of the hedgehog family expressed during odontogenesis. Expression of the *SHH* gene is mandatory for inducing the cervical loops. Signaling from the primary enamel knots initiates the secondary enamel knots, positioned at the future cusp tips, which continue to express a similar arsenal of signaling molecules. This perpetuates reciprocal epithelial-mesenchymal communication (Thesleff, 2003).

Dentin

On a weight basis, dentin constitutes approximately 70% mineral and 20% organic material; on a volume basis, approximately 50% and 30%. The remainder is water. Thus, dentin has a somewhat higher concentration of minerals than bone tissue. Bone and dentin have similar physical and chemical properties. A significant morphological difference, however, is that in bone, a proportion of the osteoblasts are embedded in the bone tissue while the dentin matrix only contains the processes of the odontoblasts. Dentin is a complex tissue presenting with several morphologically different types of calcified tissues. Primary dentin is produced at a high rate during tooth formation and constitutes the major part.

Formation

Dentin is derived from mesenchymal cells of the dental papilla. Before terminal differentiation, the papilla cells synthesize type I, type I trimer, and type III collagen, glycosaminoglycans, glycoproteins, and proteoglycans. Odontoblast differentiation is characterized by the exit of cells from the cell cycle and the subsequent polarization and secretion of predentin (Ruch et al., 1995). During functional differentiation, a change in the composition of collagen occurs as the secretion of collagen type I and type I trimer increases while the synthesis of collagen type III decreases. Synthesis of phosphoproteins, glycoproteins, glycosaminoglycans, fibronectin, and proteoglycans occurs simultaneously (Linde & Goldberg, 1993). Functional odontoblasts align in a single layer of columnar cells on the periphery of the pulp.

Structure

First, the functional odontoblasts secrete a layer of uncalcified matrix, the predentin. The predentin is subsequently mineralized to form dentin. The first layer to be deposited, the mantle dentin, located adjacent to the enamel, is the first to be calcified (Fig. 3). The more centrally located dentin is called circumpulpal dentin. As the dentin matrix is deposited, the odontoblast cell body is pushed away from the dentin surface (Butler & Ritchie, 1995)

Collagens in dentin

In dentin, collagen type I is the predominant type of collagen. Lesser amounts of collagen type III, V, and VI are also found. Procollagen is mainly secreted from the portion of the odontoblastic process located in the predentin (Weinstock & Leblond, 1974, Rabie & Veis, 1995).

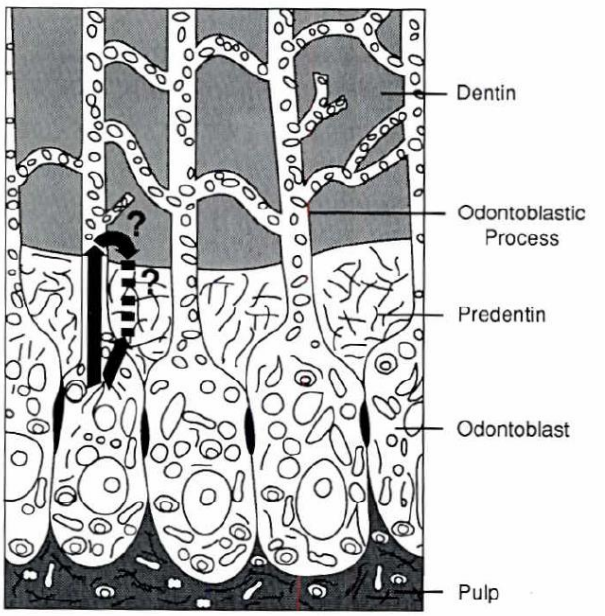

Figure 3. Odontoblasts secreting predentin, composed principally of collagen type I. The predentin is later mineralized by secretion of non-collagenous proteins. The exact routes of secretion are not fully elucidated. From Butler and Ritchie (1995).

After exocytosis of the procollagen molecules into the ECM, the N- and C-terminal propeptides of the molecules are cleaved. After collagen is secreted, there is a delay before mineralization. Mineralization is initiated in the "empty" spaces within the collagen fibrils as inorganic calcium phosphate ions are deposited within and around the fibrils. There is an intricate interaction between the hydroxyapatite crystals and collagen fibrils, where the collagen network acts like a framework but also limits the amount of mineral that can be deposited. The non-collagenous proteins are important during this process.

Non-collagenous proteins

The non-collagenous proteins of dentin consist of osteocalcin, osteonectin, and the family of small integrin-binding ligand, N-linked glycoproteins (SIBLINGs) (MacDougall et al., 2006). Osteopontin, bone sialoprotein, matrix extracellular phosphoglycoprotein, dentin matrix protein, and dentin sialophosphoprotein (DSPP) all belong to the SIBLINGs (MacDougall, 2003, MacDougall et al., 2006). DSPP is encoded by the *DSPP* gene (4q22.1). Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are the two major non-collagenous proteins formed from the initial translated propetide (Barron et al., 2008). It has been suggested that DPP has a crucial role in the nucleation of hydroxyapatite crystallites. After cleavage it moves to the mineralization front where it interacts with collagen type I (Butler, 1998). The exact mechanisms of DSP function remain to be elucidated. Proteoglycans secreted by the odontoblasts interact with collagen type I during fibril formation and may be important for

regulating the size and orientation of the fibrils. These proteins may also have a role in the timing and the site of mineralization due to their ability to retain calcium and shield mineral nucleation sites in the predentin matrix.

Dentinogenesis imperfecta

DGI is a hereditary dentin dysplasia that occurs due to an abnormal formation of dentin. The diseases have traditionally been classified according to Shields et al. (1973). DGI type I was the syndromic form associated with OI, and DGI types II and III were isolated forms. Over time, the Shield classification has become insufficient to describe the spectrum of clinical and radiographic phenotypes; some individuals present with overlapping phenotypes. The identification of the genetic cause, located in the *DSPP* gene (4q22.1) (Xiao et al., 2001, Zhang et al., 2001), have further stressed the need to update the classification system. In more recent publications, it was concluded that the same mutation in *DSPP* caused an overlapping phenotype of DGI-II and DGI-III and it was proposed that these entities were not distinct diseases, but a phenotypic variation of the same disease (Kim et al., 2005). A more recent publication has proposed a new classification of the hereditary dentin dysplasias including clinical, radiographic, and genetic findings (Table 3) (de La Dure-Molla et al., 2015).

Table 3. Classification of hereditary dentin dysplasias according to Shields et al. (1973) and de La Dure-Molla et al. (2015). Dentinogenesis imperfecta type I remains a condition associated with osteogenesis imperfecta.

OMIM*	Shield classification of isolated dentin diseases	Proposed classification of isolated dentin diseases
125400	Dentin dysplasia type I	Radicular dentin dysplasia Dentinogenesis imperfecta
125420	Dentin dysplasia type II	Mild form
125490	Dentinogenesis imperfecta type II	Moderate form
125500	Dentinogenesis imperfecta type III	Severe form

*OMIM = Online Mendelian Inheritance in Man

DGI-I

Clinical findings

Clinically, teeth affected by DGI-I present with a spectrum of hues varying from opalescent with a gray-blue hue to yellow-brown with an amber hue (Shields et al., 1973, Ranta et al., 1993, O'Connell & Marini, 1999, Barron et al., 2008). Extensive inter- and intraindividual

variations in the phenotypic expression of the colors can occur in both dentitions (Levin et al., 1980, O'Connell & Marini, 1999, Malmgren & Norgren, 2002). The deciduous dentition is more severely affected, but the cause is unknown (Fig. 4). The enamel has normal structure and mineral content but is easily dislodged due to the soft underlying dentin. The exposed dysplastic dentin is prone to severe and rapid attrition.

Radiographic findings

Pathognomonic radiographic manifestations are seen in DGI. These include abnormalities in crown shape, cervical constrictions, short constricted roots, and abnormally large or calcified pulp chambers (Shields et al., 1973, Lukinmaa et al., 1987b, Malmgren & Norgren, 2002) . Onset of pulp obliteration has been observed to occur before the teeth have erupted, indicating that the process is due to the disease and not mechanical stress.

Histologic findings

Histologic findings in teeth affected by DGI differ between individuals. The enamel is normal in structure, and the dentin-enamel junction may be flattened or scalloped. Dysplastic dentin expression includes varying degrees of sparse, thin, and irregular dentin tubules; variations in dentin tubuli width and branching; hyaline dentin void of dentin tubuli; dentin layering; and presence of cell lacunae in the body of dentin (Malmgren & Lindskog, 2003). Peripheral mantle dentin is often less severely dysplastic compared to the circumpulpal dentin, which is formed later. Dentin layering occurs in incremental lines with irregular amounts of collagen. Canal-like structures containing amorphous material and cellular inclusions are frequent findings in more severely affected teeth (Sunderland & Smith, 1980, Lukinmaa et al., 1987a, Waltimo et al., 1996, Malmgren & Lindskog, 2003).

Figure 4. Clinical findings in a 13-year-old girl with OI type I. The permanent mandibular incisors are the teeth that are most severely affected. Anterior crossbite and unilateral open bite are seen. Several permanent tooth germs are congenitally missing.

Differences between DGI-I and DGI-II

Clinical, radiographic, and histologic findings in DGI-I and DGI-II are similar, and thus challenging to differentiate (Fig. 5). The main differences include presence of OI in DGI-I. A more extensive intraindividual variability of expression is found in DGI-I and an almost complete penetrance is seen in DGI-II (Witkop, 1971, Shields et al., 1973). However, the genetic causes are different between the two types. Mutations in *COL1A1* and *COL1A2* give rise to DGI-I (Pallos et al., 2001), and a mutation in the *DSPP* gene (4q22.1), to DGI-II (Xiao et al., 2001, Zhang et al., 2001).

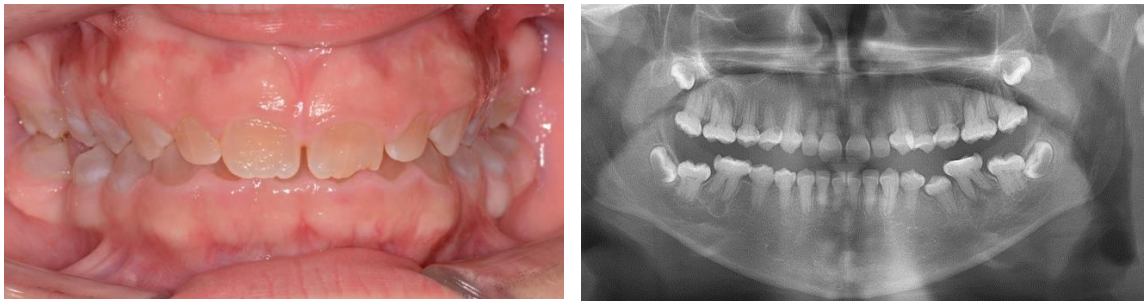

Figure 5. Clinical and radiographic findings in an 11-year-old boy with a severe form of DGI-II.

Differential diagnosis of DGI

The clinical features of several conditions are similar to those of DGI, which may cause some confusion when setting a diagnosis. These include tetracycline-induced discoloration, congenital erythropoietic porphyria, and amelogenesis imperfecta. Discoloration due to tetracycline can occur in both dentitions, and the hue, varying from grey to brown, depends on the dose and type of drug (Cheek & Heymann, 1999). Uneven discoloration occurs in congenital erythropoietic porphyria, a disease characterized by an inborn error of porphyrin metabolism (Bhavasar et al., 2011). In Vitamin D-dependent rickets, clinical signs include a yellow-brown discoloration radiographically confirmable periodontal disease, large pulp chambers, and short roots. Vitamin D-resistant rickets is characterized by attrition and abscesses around non-carious teeth (Goodman et al., 1998). DGI features also occur in Ehlers-Danlos syndrome, Schimke immune-osseous dysplasia, brachio-skeleto-genital syndrome, and Goldblatt syndrome (Barron et al., 2008)

Prevalence of dentinogenesis imperfecta type II

Information on the prevalence and incidence of DGI-II is scarce. The earliest investigation estimated an incidence of 0.013–0.017% in a cohort of families in the state of Michigan in 1957 (Witkop, 1957). Gupta et al. (2011) found that DGI was the rarest developmental dental

anomaly, presenting in 0.09% of the individuals in an Indian population. The prevalence of type II in a French population was estimated to be 0.057% (Cassia et al., 2017) and 0.1% in Saudi children (Yassin, 2016). The Indian, French, and Saudi Arabian studies were single center cohorts and had retrospective designs. Based on clinical experience, and the design of previous investigations, there is reason to believe that the prevalence of DGI-II is lower in Swedish children and adolescents.

Osteogenesis imperfecta and dental aberrations

Oral manifestations, with DGI being the most pronounced, are common findings in OI. Bone and dentin share many characteristics and are very similar in structure. Type I collagen is the main organic component in both tissues. A limited number of studies have investigated dental aberrations in OI. Aside from DGI, tooth agenesis, denticles, invaginations, taurodontism, and impacted permanent second molars have been found in OI (Bauze et al., 1975, Paterson et al., 1983, Sunderland & Smith, 1980, Schwartz & Tsipouras, 1984, Lukinmaa et al., 1987b, Lund et al., 1998, O'Connell & Marini, 1999, Malmgren & Norgren, 2002, Saeves et al., 2009). Aberrant craniofacial development is another feature frequently seen in OI (Isshiki, 1966, Stenvik et al., 1985, Ormiston & Tideman, 1995, Jensen & Lund, 1997, Malmgren & Norgren, 2002, Waltimo-Siren et al., 2005, Chang et al., 2007, Jabbour et al., 2018, Rizkallah et al., 2013).

Dentinogenesis imperfecta

DGI was established as one of the cardinal signs of OI nearly 50 years ago (Witkop, 1971, Sillence et al., 1979). The Paterson et al. (1983) study allocated participants to a group without DGI (OI type IA) or a group presenting with DGI (OI type IB). DGI occurs more frequently in OI type III compared to the milder types (Lund et al., 1998, O'Connell & Marini, 1999, Malmgren & Norgren, 2002). In the early studies, information on the diagnostic criteria for DGI was scarce (Bauze et al., 1975, Paterson et al., 1983) (Table 4). It was later found that a diagnosis of DGI cannot be based on clinical signs only. Some individuals only present with radiographic and/or histologic findings (Lukinmaa et al., 1987a). Discolored teeth and histopathological findings in dentin sometimes occur in the absence of bulbous crowns, cervical constriction, and obliteration. Teeth from the same individual are almost identical, and intra-familial variability is low (Malmgren & Lindskog, 2003, Lukinmaa et al., 1987a). Nearly total accordance regarding DGI has been found between affected children and parents (Malmgren & Norgren, 2002). Presence of DGI has also been found to be more common in the deciduous dentition than the permanent (O'Connell & Marini, 1999, Malmgren & Norgren,

2002); it is not at all sure that a child with DGI in the deciduous dentition will develop DGI in the permanent dentition. Why this is so is not known.

Table 4. Summary of prevalence studies on dentinogenesis imperfecta (DGI) in osteogenesis imperfecta (OI).

Study	Type I		Type III		Type IV		Total	
	OI, n	DGI, n	OI, n	DGI, n	OI, n	DGI, n	OI, n	DGI
Bauze et al. (1975)	22	1 (5%)	17	7 (41%)	3	0	42	19%
Paterson et al. (1983)	166	29	-	-	-	-	166	17%
Paterson et al. (1983)	69	41%	-	-	48	69%		
Schwartz & Tsipouras (1984)	20	8 (40%)	7	4 (43%)	1	1 (100%)	28	43%
Lukinmaa (1987)	45	4 (9%)	2	1 (50%)	16	13 (81%)	68	32%
Lund et al. (1998)	50	4 (8%)	16	13 (81%)	22	8 (37%)	88	28%
O´Connell & Marini (1999)	-		22	18 (82%)	18	11 (61%)	40	73%
Malmgren & Norgren (2002)	36	10 (28%)	15	10 (67%)	14	7 (50%)	65	42%
Saeves et al. (2009)	74	7 (10%)	8	8 (100%)	12	2 (17%)	94	19%

Tooth agenesis

Tooth agenesis denotes congenital absence of one or more teeth. It is one of the most common developmental orofacial birth defects. Hypodontia is defined as < 6 missing tooth germs (excluding third molars), and oligodontia as agenesis of ≥ 6 permanent teeth. Anodontia is the complete lack of tooth development in the deciduous and permanent dentitions (Phan et al., 2016). Tooth agenesis has been found in individuals with OI (Lukinmaa et al., 1987b, Malmgren & Norgren, 2002). In the Lukinmaa et al. study, most patients were diagnosed with hypodontia. Seven of the nine lacked only one permanent tooth, and the others lacked several teeth. The prevalence of hypodontia was 18.4% in this cohort. No data regarding type of missing tooth germs were presented. Malmgren and Norgren (2002) found a similar frequency of tooth agenesis, 22%.

Taurodontism

Taurodontism is defined by a lack of cervical constriction at the level of the cemento-enamel junction (CEJ), an enlarged pulp chamber, and apical displacement of the pulpal floor (Jafarzadeh et al., 2008). Taurodontism is a rare finding in the general population, where it occurs in 0.3–2.5% (Backman & Wahlin, 2001, Gupta et al., 2011). Presence of taurodontism has been identified in OI. Lukinmaa et al. (1987) diagnosed the trait in 6% of their investigated individuals. A later study found a higher prevalence, 42%, among individuals with OI type I and IV, but not in OI type III (Malmgren & Norgren, 2002). The etiology of the condition is not fully elucidated. Five causes have been proposed: (1) a specialized or retrograde characteristic, (2) a primitive pattern, (3) a Mendelian recessive trait, (4) an atavistic feature, and (5) a mutation resulting from odontoblastic deficiency during dentinogenesis of the roots. Hamner et al. suggested that Hertwig's epithelial root sheath (HERS) explained the morphology that is characteristic for taurodontism (Hamner et al., 1964).

Retention of permanent second molars

Retention of permanent second molars is an unusual finding in healthy individuals, for example, Bondemark and Tsiopa (2007) observed retention in only 0.6% of healthy adolescents (Bondemark & Tsiopa, 2007). However, impaction of second permanent molars, predominantly in the upper jaw, was found in one study in 37% of individuals with OI (Malmgren & Norgren, 2002).

Tooth eruption failure is primarily of two types: (1) Impaction, where retention is due to the ectopic position of the tooth germ or to an obstacle in the eruption path, and (2) Primary retention, where cessation of eruption is not caused by a physical obstacle in the eruption path or by the ectopic position of the tooth germ (Raghoebar et al., 1991). The etiology of permanent second molar retention includes aberrant craniofacial development, systemic disease, crowding, and disturbances of the PDL and nerve supply. Heredity may also contribute (Magnusson & Kjellberg, 2009).

Malocclusion

A common feature in OI is abnormal craniofacial development, which includes Class III malocclusions, anterior and posterior crossbites, and open bites (Jensen & Lund, 1997, Malmgren & Norgren, 2002, Waltimo-Siren et al., 2005, Chang et al., 2007, Rizkallah et al., 2013). Inhibition of maxillary growth, maxillary hypoplasia in the anteroposterior and vertical dimensions, and mandibular protrusion and hyperplasia are all possible explanations of the frequently occurring Class III malocclusions (Isshiki, 1966, Stenvik et al., 1985, Ormiston & Tideman, 1995).

Genotype-phenotype correlation

Few earlier studies have evaluated dental genotype-phenotype associations. The most relevant ones emphasized presence of DGI (Wenstrup et al., 1990, Jensen & Lund, 1997, Lund et al., 1998, Luder et al., 1996, Rauch et al., 2010, Lindahl et al., 2015) and craniofacial development including malocclusion (Jensen & Lund, 1997, Jabbour et al., 2018). Lund et al. (1998) was the first study to evaluate a larger cohort. DGI is significantly more common in individuals presenting with mutations inducing a qualitatively changed collagen type I (Wenstrup et al., 1990, Lund et al., 1998, Luder et al., 1996, Rauch et al., 2010, Lindahl et al., 2015).

In addition, Lund et al. proposed that DGI could be a marker of structurally abnormal type I collagen (Lund et al., 1998). This research group also found that 55% of the individuals with mutations in the collagen α1(I) chain had DGI, whereas the corresponding figure was 80% among those with an α2(I) mutation. The Luder et al. case report (1996) examined an extracted permanent canine from a patient with OI type III and a mutation in *COL1A2*. The clinical and radiographic findings of the primary teeth agreed with DGI. The histological appearance of the permanent teeth revealed mild aberrations while the OI type was severe. It was hypothesized that odontoblasts could compensate for the genetic defect by excluding α2(I) chains and forming α1(I) homotrimer chains (Luder et al., 1996). Later studies revealed that the position is important for the presence of DGI, where DGI is more common in individuals harboring mutations in the C-terminal portion of the α chains (Rauch et al., 2010, Lindahl et al., 2015). In the Lindahl et al. study, a DGI diagnosis was based solely on primarily clinical findings, while the Rauch et al. study presented no information on how the diagnosis was made or if the deciduous, permanent, or both dentitions were examined.

Genetic data of individuals with OI presenting with oral manifestations has been scarce in the literature. As DGI type I and the other dental aberrations have the most varying phenotypic expressivity, further evaluation of the dental aberrations in relation to the characteristics of the mutation is necessary. Greater understanding would increase our ability to better predict the severity and progress of the disease. Furthermore, this knowledge could improve our options to individualize dental treatment of children and adolescents with OI as genetic analysis may be a helpful aid in identifying individuals with an increased risk of oral complications.

AIMS OF THE THESIS

General aim

The general aim of the present thesis was to study the breadth of the spectrum of oral manifestations in a large cohort of children and adolescents with OI and relate it to genetic findings to enable better identification of individuals presenting with risk of severe oral manifestations. A further aim was to investigate the prevalence of DGI type II in Swedish children and adolescents, in order to map the extent of this rare disease in Sweden.

Specific aims

Study I

To test the hypothesis that the prevalence of DGI type II is significantly lower than previously reported among Swedish children and adolescents and that there are undiagnosed cases of OI in individuals who were incorrectly diagnosed with DGI type II.

Study II

To test the hypothesis that congenitally missing teeth are more prevalent in individuals presenting with a mutation in *COL1A1* and *COL1A2* that is predicted to cause a qualitatively defect collagen type I and is related to type of OI and presence of DGI.

Study III

To test the hypothesis that presence of DGI in both dentitions, taurodontism, and retention of permanent second molars are more prevalent in children and adolescents with mutations predicted to induce qualitative defects of collagen type I.

Study IV

To test the hypothesis that individuals with OI, oligodontia, and severe hypodontia also presented with variants in other genes related to tooth development; that is, that these individuals presented with variants in modifying genes related to both *COL1A1*/*COL1A2* and tooth development.

SUBJECTS AND METHODS

Subjects

All cohort individuals in studies II, III, and IV received care from the Swedish national multidisciplinary pediatric OI team at Astrid Lindgren Children's Hospital at Karolinska University Hospital in Stockholm. Today the team has several specialists involved in the assessment and care of children and adolescents with OI. The specialists represent the following fields: neurology, orthopedic surgery, genetics, radiology, orthopedic engineering, physiotherapy, occupational therapy, pediatric nursing, nursing, and pediatric dentistry. The team has assessed more than 90% of all Swedish children and adolescents with OI. All children seen by the team receive an individualized assessment. The pediatric OI team collaborates closely with local medical, dental, and habilitation teams.

Since its formation in 1991, the pediatric dentists on the team have assessed 263 individuals. Of those, 179 were asked between 2006 and 2014 to participate in studies on genotype-phenotype correlations in OI. The individuals in Studies II, III, and IV all come from this cohort. Studies II and III were a continuation of a previous study (Lindahl et al., 2015) and focused on oral manifestations of OI (Fig. 6).

Study I

At the 2013 Swedish Academy of Pediatric Dentistry meeting, we informed all pediatric dental specialists and dentists in Sweden of the planned study, and when we initiated it in 2014, invited them (n=179) to participate (all public and private specialist pediatric dental clinics [n=47] in all Swedish counties [n=21]). We asked for reports on all children and adolescents (0.1–19 years old) who had been diagnosed with DGI-II and who were or previously had been in treatment. During 2014–2017, we continuously followed up all cases in order to confirm all children and adolescents presenting with DGI-II.

Study II

We evaluated 128 children and adolescents with OI (n = 128; boys = 74, girls = 54). Only one member from each family (the member with the most complete data and aged ≥ 7 years, when all permanent tooth germs could be evaluated) were accepted. We retrieved clinical characteristics from the medical and dental records, including information on the deciduous dentition and type of OI according to Sillence et al. (1979). OI type I was present in 81 individuals, type III in 17, and type IV in 30.

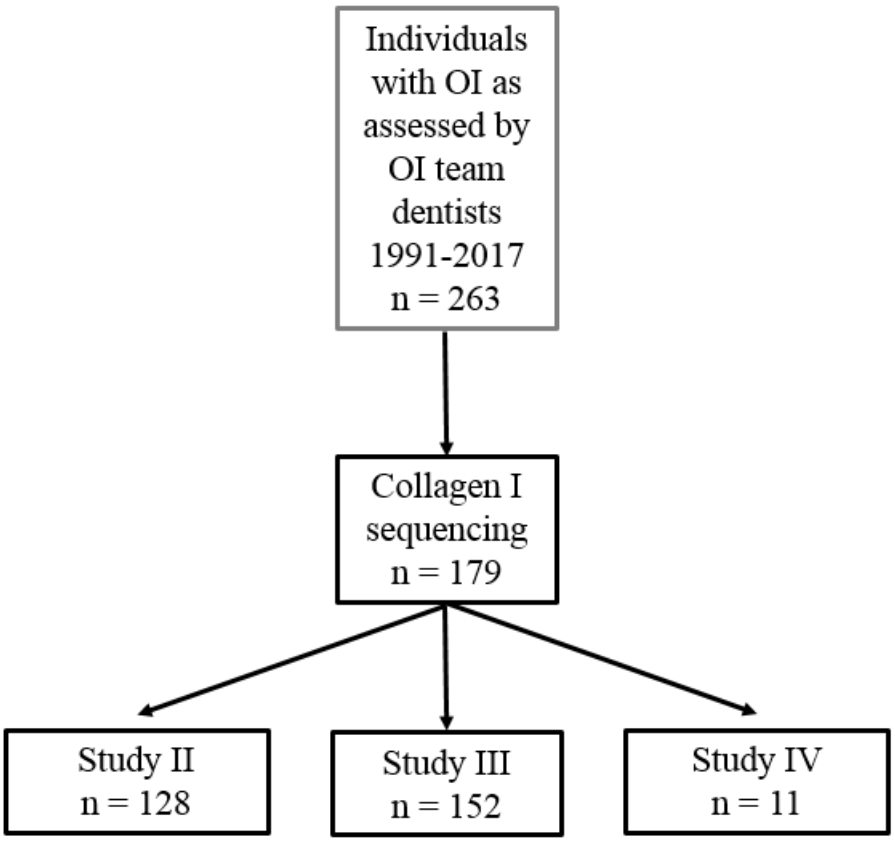

Figure 6. Flow chart illustrating the study cohort of children and adolescents with OI whom OI team pediatric dentists evaluated regularly during 1991–2017. All 179 invited individuals consented to participate in genotype-phenotype investigations.

Study III

Study III recruited children and adolescents with OI from the same cohort as Study II. To avoid skewing the genetic impact of each mutation, this study also included only one child per family, the child with the most complete clinical data. In contrast to Study I, this study had no inclusion criterion for age. Any family history of DGI was noted. Children with no erupted teeth were excluded. The final study group comprised 152 individuals, 67 females and 85 males, of whom 63% (96) were classified as OI type I, 15% (22) as OI type III, and 22% (34) as OI type IV.

Study IV

This study cohort included 11 children and adolescents with OI who had participated in a previous study (Malmgren et al., 2017): 7 presenting with oligodontia; 2 presenting with hypodontia; 1 with OI type IV who had the same variant in *COL1A1* as another participant with oligodontia; and a participant from this previous study who had OI type IV but who was newly diagnosed with oligodontia. We evaluated the cohort further genetically. Three

participants were classified as OI type I, four as OI type III, and four as OI type IV. To allow evaluation of all permanent teeth, one inclusion criterion was age ≥ 7 years.

Ethical permission

The Swedish regional ethics committees in Stockholm and Uppsala approved the study protocols (Daybook no. 157/99, 2014/254-31, 2012/2106-31/4, and Ups 2006/212. Recruited participants and/or their legal guardians signed informed-consent forms.

Clinical and radiographic examinations (I)

At the start of this study, we contacted all public and private pediatric dental clinics in Sweden and requested all children and adolescents who had been diagnosed with DGI-II at the clinics and who were or previously had been in treatment. We asked the practitioners to examine the individuals and document the presence of DGI with photographs. Furthermore, we asked for available radiographs, to enable confirmation of DGI.

Using the structured questionnaire and examination protocol that we sent them, the pediatric dentists interviewed and examined all patients with DGI-II regarding medical aspects such as bruising, prolonged bleeding, spraining, fractures, hearing impairment, and family history of osteoporosis and OI. The hue of the sclerae, the whites of the eyes, was assessed. The examiners used the Beighton scale to evaluate joint hypermobility; the hypermobility score ranged from 0 to 9. The examiner explained and demonstrated each maneuver before asking the patient to attempt it.

The clinical and, when indicated, panoramic radiographic examinations assessed dental variables associated with OI. Clinically, the following signs were evaluated: retained teeth (failure to erupt), malocclusion, and DGI (as defined by pathologic discoloration, attrition, and fractures). Radiographically, we assessed number of tooth germs, extended pulp chambers (taurodontism), and DGI indicators (bulbous crowns with cervical constriction, pulpal obliteration, and short roots). Individuals with suspected OI were referred to the OI pediatric team at Astrid Lindgren Children's Hospital for further examination.

Clinical and radiographic examinations (II, IV)

All dental examinations were performed at Astrid Lindgren Children's Hospital, Eastman Institute, or Karolinska Institutet in Stockholm. Clinical characteristics were collected from medical and dental records, including information regarding the deciduous dentition and the type of OI according to Sillence et al. (1979). Panoramic radiographs were analyzed regarding DGI and number of permanent teeth and tooth germs. The radiographic evaluation of DGI

included recording abnormalities in crown shape, cervical constrictions, and abnormally large or calcified pulp chambers. In cases of dental agenesis, a detailed family history was made.

Clinical and radiographic examinations (III)

Dentinogenesis imperfecta

We examined the participants clinically if they had not been previously examined at Astrid Lindgren children's hospital, Eastman Dental Institute or Karolinska Institutet. Signs of DGI were noted in the patient history during the clinical examination. Characteristic grey-blue or yellow-brown discoloration of deciduous or permanent tooth crowns solely or in combination with pathological attrition and/or fractures were regarded as clinical signs of DGI. Photographs were made to aid in further evaluation.

All available radiographs, were evaluated for signs of DGI: abnormalities in crown shape, cervical constrictions, and abnormally large or calcified pulp chambers. We based our diagnosis of DGI on the combined clinical, radiographic, and histological findings. Two authors (KA and BM) evaluated all individuals retrospectively to document the development of the dental phenotype.

Taurodontism

We assessed the lower first permanent molars for signs of taurodontism. The degree of the relative amount of apical displacement was based on the size of the pulp chamber and the size and shape of the roots (Shaw, 1928) (Fig. 7). As taurodontism cannot be assessed in teeth exhibiting radiographic DGI characteristics, we excluded individuals with DGI from further evaluation of taurodontism.

Retention of permanent second molars

We assessed permanent second molars as retained based on radiographic signs and clinical findings, or absence of eruption at age 15 years. Inclination of retained second molars was assessed as mesioangular, vertical, or distoangular.

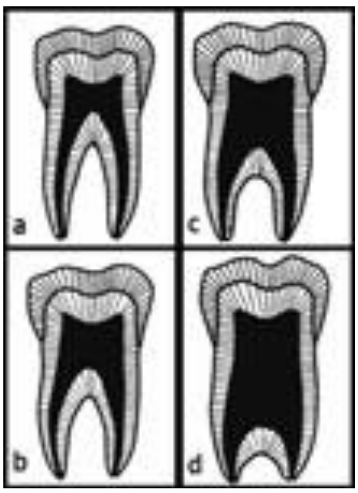

Figure 7. Classification of taurodontism: (a) cynodont = morphologically normal molar, (b) hypotaurodont = moderate enlargement of the pulp chamber, (c) mesotaurodont = extensive enlargement of the pulp chamber with shorter, but still separated roots, and (d) hypertaurodont = severe form, where the pulp chamber almost reaches the apex before it exits into 2 or 4 channels (Shaw 1928).

We used lateral cephalometric radiographs to relate retention of permanent second molars to the craniofacial position of the jaws, type of OI, presence or absence of DGI, and type of mutation. Facad® software for orthodontic tracing and cephalometric analysis (ILEXIS AB, Linköping, Sweden) analyzed the lateral cephalograms. The positions of the maxilla and mandible were compared to normative cephalometric data for a Swedish population matched according to age, gender, and normal occlusion (Thilander et al., 2005). The sella–nasion–A point (SNA) angle was used to determine the position of the maxilla in the anterior-posterior direction, and the SN/SpPm angle to evaluate the position of the maxilla in the vertical direction. The sella–nasion–B point (SNB) angle was used to determine the anterior-posterior position of the mandible. We determined the inclination of the mandible by plotting the angle between the mandibular line (ML) and the SN line. We also measured the inclination of the upper and lower incisors based on the long axis of the teeth by their relation to the SN line and the ML (Fig. 8).

Clinical and radiographic examination (IV)

All participants underwent a detailed clinical and radiographic evaluation regarding total number of permanent tooth germs and teeth. In cases of dental agenesis, a family history was taken. The clinical examination included recording signs of DGI.

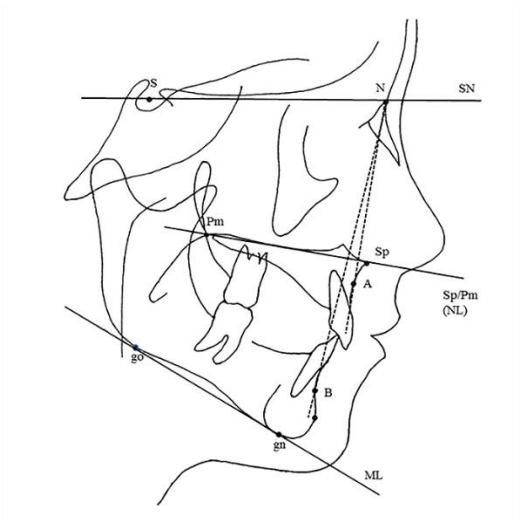

Figure 8. Skeletal reference points, lines, and angles evaluated. Reference points: S: The center of sella turcica, N: Nasion, Sp: Spina, the apex of the anterior nasal spine, Pm: Pterygomaxillare, A: The most concave point of anterior maxilla, B: The most concave point on the mandibular symphysis, Gn: Gnathion, the lowest point in the lower border of the mandible in the median plane, Go: Gonion, the most posterior inferior point on angle of the mandible. Reference lines: SN, SpPm (NL), ML: The mandibular line. Reference angles: SNA, SNB, SN/SpPm, SN/ML.

DNA samples (II, III)

Venous blood samples were taken and analyzed at Uppsala University Laboratory. DNA was extracted from peripheral blood, and all exon-intron boundaries of the collagen type I genes, *COL1A1* and *COL1A2* were amplified using standard protocols and primers. Sanger sequencing of DNA was done. Identified variants were confirmed by resequencing the affected exon, and when necessary, a segregation analysis in family members was done.

DNA samples (IV)

Venous blood samples were collected from all individuals. Genomic DNA was isolated at the Clinical Genetics Unit of Karolinska University Hospital.

DNA isolation (II, III)

Leucocyte DNA was isolated from peripheral blood using the Wizard® Genomic DNA Purification Kit (Promega) per manufacturer instructions. The GeneElute™ Mammalian Genomic DNA miniprep Kit (Sigma) was used to isolate DNA from bone and peripheral blood from infants. DNA concentration was determined with the Nanodrop ND-1000 (NanoDrop Technologies, USA).

Polymerase chain reaction and DNA sequencing (II, III)

Polymerase chain reaction (PCR) primers were designed to cover the exon and flanking introns of the *COL1A1* and *COL1A2* genes and then carried out on a GeneAmp PCR system 9800 using AmpliTaq® Gold kits and standard reagents. An adjusted Big Dye Terminator 3.1 sequencing protocol was used for the sequencing reactions. The products were run on a 16-capillary ABI 3130xl Genetic Analyzer automated sequencer and analyzed with Seqscapre v.2.5. Applied Biosystems, CA, USA (www.appliedbiosystems.com) supplied all reagents, equipment, and software.

Multiplex ligation-dependent probe amplification (II, III)

Individuals found to be negative for mutations after Sanger sequencing underwent multiplex ligation-dependent probe amplification (MLPA) analysis of *COL1A1* and *COL1A2* in order to detect large deletions and insertions. We used SALSA-MLPA Kits P271 *COL1A1* and P272 *COL1A2* (MRC-Holland, Holland) per manufacturer instructions (www.mlpa.com).

PCR and Sanger sequencing (IV)

PCR and Sanger sequencing were performed to validate variants of interest and for segregation analysis. 100 ng of genomic DNA was mixed with 2.5 microliter of 10X PCR Buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µmolar forward and reverse primer and 2U of Platinum^{TM} Taq DNA Polymerase (Invitrogen). The reaction was incubated at 95°C for 2 minutes and followed by 35 cycles at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 60 seconds. A final incubation of 2 minutes at 72°C was performed. PCR products were run on 2% agarose gel and then cleaned up for cycle sequencing using illustra ExoProStar 1-Step (GE Healthcare Life Sciences). Cycle sequencing reactions were done using with BigDye Terminator v3.1 (Applied Biosystems) and M13 primers. After ethanol precipitation to clean up unbound ddNTPs, fragments were sequenced in the ABI 3730XL Genetic Analyzer (Applied Biosystems). Primers for exon 6 of *AXIN2* are available upon request.

Whole-genome sequencing and bioinformatics analysis (IV)

Libraries were prepared for sequencing on Illumina HiSeqX (Illumina Inc, San Diego, CA, USA) from the genomic DNA using the Illumina TruSeq DNA PCR-Free kit with a mean insert size of 400 bp. An in-house pipeline developed by the Science for Life Laboratory, Stockholm, Sweden was used to map reads to the human reference genome (hg19) and to call variants. Data were aligned to the reference genome using bwa (v0.7.12).

We deduplicated, recalibrated, and indel realigned raw alignments using GATK (v3.3-0-geee94ec). The quality control information was gathered using Qualimap (v2.2) and single nucleotide variants (SNVs) and indels were called using the HaplotypeCaller in GATK. We further processed the variants with GenotypeGVCFs, VariantRecalibrator, ApplyRecalibration, VariantFiltration and SelectVariants tools in GATK (v3.7); the variants were then functionally annotated using the Variant Effect Predictor (VEP; version 89) and loaded into a database using GEMINI (v0.20.0). Two approaches were followed. In the first approach, we excluded variants with minor allele frequencies of 0.1% and higher in the 1000 Genomes Project (1000G), the 6500 NHLBI-GO Exome Sequencing Project (ESP), the Swedish Genome Project (SweGen), and the Exome and Genome Aggregation Consortium (ExAC and gnomAD). In the second approach, we focused only on genes involved in human nonsyndromic and syndromic hypodontia/oligodontia and tooth development in animal models. Here, we applied a minor allele frequency higher than 1% for the listed genome projects to filter out common variants. In both approaches, variants located in repetitive sequence regions were excluded. Only non-synonymous variants, frameshift indels and putative splice site variants were considered for further analysis. We used Combined Annotation Dependent Depletion (CADD) to score the pathogenicity of the SNVs. The evolutionary conservation of variants was evaluated with Genomic Evolutionary Rate Profiling (GERP). The variants were explored in the database using built-in tools in GEMINI and were visualized on Integrated Genome Viewer (IGV).

Structural variants were analyzed using the FindSV pipeline (https://github.com/J35P312/FindSV), which merges calls from CNVnator V0.3.2 and TIDDIT. The structural variants were annotated using the VEP and filtered based on the quality flag of the variant. The filtered and annotated variants were then sorted based on a local structural variant frequency database consisting of variants from 1000 healthy individuals in the SweGen project. The reads at breakpoints were visualized in the IGV. The structural variants that fell into intergenic regions as well as intronic deletions and duplications were excluded. We focused only on breakpoints that were located in the exons of the coding genes listed in our gene list.

Nomenclature (II, III, IV)

We followed the recommendations of the Nomenclature Committee of the Human Genome Variation Society (www.HGVS.org/varnomen) to describe sequence variations and used the GenBank reference sequences of *COL1A1* (genomic DNA NG_007400.1 and cDNA NM_000088.3) and *COL1A2* (genomic DNA NG_007405.1 and cDNA NM_000089.3).

Collagen type I mutations were reported to the osteogenesis imperfecta & Ehlers-Danlos syndrome variant database (http://www.le.ac.uk/genetics/collagen/) (Dalgleish, 1997, Dalgleish, 1998). Study IV used the most recent GenBank reference sequences for the relevant gene to describe the variants.

Statistical analyses

All statistical analyses in Study II were done using Statistica v. 12.5 (Statsoft, Scandinavia AB, Uppsala Sweden). The statistical analyses in Studies I, III, and IV were done using the Statistical Package for the Social Sciences (SPSS for Windows, v. 24 and v.25; IBM SPSS Inc., Chicago, IL, USA).

Study I

Prevalence was calculated as the ratio of the number of individuals with DGI to the number of individuals born between 1996 and 2015 in Sweden. Cumulative incidence was calculated as the ratio of the number of new cases of DGI reported during 1996–2015 to the number of newborns at risk of DGI during that time period. Incidence rate per unit of time was calculated as the ratio of the number of new cases to the number of persons at risk of developing DGI. Data were summarized as proportions, or counts, or means and standard deviations. For categorical variables, the X^2 test determined differences in frequencies of dental aberrations. The independent *t*-test evaluated continuous variables. Two-tailed *p*-values were computed using $p < 0.05$ to denote a significant deviation from the null hypothesis.

Studies II and III

We used Fischer's exact test (X^2 test) to evaluate categorical variables and determine differences in frequencies of tooth agenesis, presence of DGI, taurodontism, retention of permanent second molars, malocclusion, quantitative/qualitative collagen I defects, genes (*COL1A1* vs. *COL1A2*), gender, and type of OI. Two-tailed *p*-values were used, and $p < 0.05$ denoted a significant deviation from the null hypothesis. The independent t-test was used for evaluation of continuous variables.

We chose age- and gender-matched controls to assess deviations in the cephalometric measurements of individuals with OI. Differences were calculated using this formula: Dev = (Xi-$\bar{X}$ /sd) where Dev = difference, Xi = actual value, $\bar{X}$ = average value of the normal material, and sd = standard deviation of the reference material. The Mann-Whitney U test assessed differences between permanent second molar retention, DGI, cephalometric values, and Pamidronate treatment.

Study IV

SPSS for Windows, v.25; IBM SPSS Inc., Chicago, IL, USA) was used for calculations of age. Otherwise statistical analyses were not applicable.

RESULTS

Point prevalence, cumulative incidence, and incidence rate of DGI-II (I)

The response rate concerning known cases was 100%; all clinics contacted us. Twelve counties reported cases of resident children diagnosed with DGI-II: 44 children and adolescents (19 males, 25 females) born between 1996 and 2015. During this period, there were 2,044,530 births in Sweden (Socialstyrelsen, 2017). The 2015 point prevalence was thus estimated as 0.0022% (95% CI, 0.0016–0.0029%; 2.2 in 100,000 individuals or 1 in 45,455 individuals). The incidence proportion/cumulative incidence of DGI-II was 0.0022% (2.2 per 100,000 newborns per 20 years) in Sweden; the incidence rate was 0.00011% (95% CI, 0.000076–0.00014%) per person-year or 1.1 individuals per 1,000,000 person-years of observation/risk.

Dental aberrations in children and adolescents with DGI-II

Thirty individuals were clinically assessed according to the examination protocol. The remaining cases (n=14) were diagnosed based on personal communication with the responsible practitioner due to the patient wishing to be excluded from further participation or inability to attend the examination.

Dental agenesis (hypodontia) was found in 9% of the children and adolescents with DGI-II. Tooth impaction/retention was diagnosed in 17% and pulpal obliteration was detected in all individuals for whom a radiographic examination was available (n=24).

Presence of variables associated with OI in individuals presenting with DGI

Variables associated with OI could be evaluated in the majority of the examined individuals. Eight subjects (25%, 6 boys and 2 girls) stated that they often had bruises and bruised easily. One individual (3%) reported a history of prolonged bleeding. Two individuals (6%) had experience of spraining. One 13-year-old girl reported a previous radius fracture (3%). The fracture was assessed as unassociated with OI as it had occurred after falling off a swing. One individual reported hearing impairment (3%) and one examined individual was found to have a scleral hue deviating toward blue or blue-grey tones (3%). Signs of joint hypermobility were found in 50% of the subjects who consented to be examined: 44% (7/16) boys and 56% (9/16)

girls. The 30 examined individuals had a mean Beighton hypermobility score of 1.6 ± 2.1 (range 0–6).

Referral for further medical evaluation of OI

Clinical and radiographic findings in one individual, a 2-year-old boy, gave rise to a suspicion of undiagnosed OI. The boy was referred to the national OI multidisciplinary pediatric team at Astrid Lindgren Children's Hospital at Karolinska University Hospital; examination confirmed an OI diagnosis for the boy and suspected OI in the mother.

Tooth agenesis related to mutations in *COL1A1 and COL1A2* (II)

Variants in *COL1A1* and *COL1A2* were identified in 104 of the 128 investigated individuals (103 by Sanger sequencing and 1 by MLPA). Of these mutations, 43% were predicted to induce a quantitatively changed collagen type I while 57% were predicted to cause a qualitatively changed protein. A large deletion of *COL1A2* involving exon 1−18 was detected by MLPA in 1 female. No variant in the collagen type I genes could be identified in 24 individuals (10 in OI type I (12%), 3 in OI type III (18%), and 11 in OI type IV (37%).

The mean age at time of clinical evaluation was 13.8±4.3 years. The radiographic examination revealed tooth agenesis in 22 of 128 individuals (17%). Of those, 11% presented with hypodontia and 6% with oligodontia. Permanent premolars were the most frequent congenitally missing teeth (91%). No individuals exhibited tooth agenesis in the deciduous dentition. We detected pathogenic variants in *COL1A1* or *COL1A2* in 16 of 22 individuals presenting with tooth agenesis. All detected variants were located within the triple-helical domain of the molecules with no correlation between amino acid position and number of missing teeth. The prevalence of glycine to serine substitutions was similar among the patients with agenesis compared with the entire cohort. Tooth agenesis was more common in individuals with DGI (p=0.016) (Fig. 9) and in those harboring mutations predicted to cause a qualitatively changed protein (13/59, 22%) compared to those with quantitative defects (3/45, 7%), but this difference was not significant. Tooth agenesis was more common in individuals with OI type III (47%), compared with children with types I (12%, p=0.003) and IV (13%, p=0.017).

Oligodontia was found in eight individuals. Six of these (75%) presented with mutations predicted to cause qualitative defects. In these individuals, gender, presence of DGI, and type of OI were equally distributed. We detected the same *COL1A1* variant, p.(Gly821Ser), c.2461G>A, in two unrelated boys with OI type IV. One with a *de novo* mutation presented with eight teeth missing and no DGI. The other boy had no tooth agenesis, but he did have

DGI, as did his father. Another variant in *COL1A1*, p.(Gly1040Ser), c.3118G>A was identified in two other unrelated individuals (a girl and a boy). The girl had seven missing permanent teeth, while the boy had none missing. Both variants were *de novo* mutations, and neither individual exhibited signs of DGI.

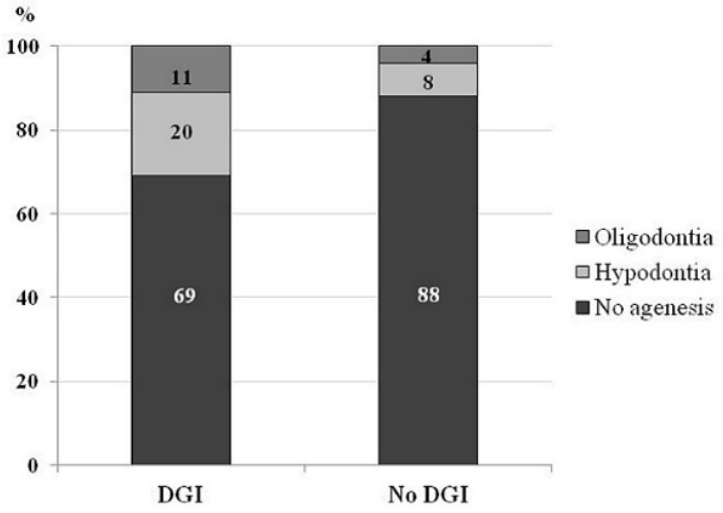

Figure 9. Distribution of tooth agenesis in relation to presence (n = 35) and absence (n = 93) of dentinogenesis imperfecta (DGI).

Dentinogenesis imperfecta, taurodontism, and retention related to mutations in *COL1A1* and *COL1A2* (III)

Mutations in COL1A1 and COL1A2

We found *COL1A1* and *COL1A2* mutations in 81% of the individuals with OI (123/152; 121 by Sanger sequencing and 2 by MLPA). Presence of DGI was seen in 70% of those with a predicted qualitatively changed protein compared to 27% in whom a quantitative defect had been found ($p<0.001$). 29% of the individuals (44/152) had a clinical and radiographic diagnosis of DGI and another 19% (29) only a histological diagnosis. In these 73 individuals, the prevalence of DGI was highest in children with OI type III and lowest in children with OI type I (86% vs. 31%; $p<0.001$). From clinical, radiological, and histological findings, we distinguished three main groups of patients with DGI: 1 = clear and distinct DGI in the deciduous dentition only, 2 = DGI in both dentitions, and 3 = DGI diagnosis possible only on histological examination.

Presence of DGI in the deciduous dentition only

Twenty individuals exhibited DGI in the deciduous dentition only. In most of these individuals, DGI was diagnosed clinically and radiographically with only subtle clinical signs in the

permanent dentition. These signs included slight discoloration of the mandibular incisors. In some cases, the diagnosis could be confirmed only by radiographic examination.

Presence of DGI in both dentitions

29% of the individuals for whom data on both dentitions were available had clear and distinct signs of DGI. Among group-2 individuals with *COL1A1* mutations who had a glycine substitution C-terminal of p.Gly305, 70% (7/10) exhibited DGI in both dentitions compared to no group-2 individual (0/7) with a mutation N-terminal of this point (*p*=0.001**)**. No individual with an N-terminal mutation exhibited clinical or radiographic signs of DGI in their deciduous teeth. In individuals with a glycine substitution located C-terminal of p.Gly211 in *COL1A2*, 80% (8/10) exhibited DGI compared to no individual (0/5) presenting with a mutation N-terminal of this point (*p*=0.007) (Fig. 10). None of the individuals with an N-terminal mutation exhibited any signs of DGI in their deciduous dentition. The congruence between presence of clinical DGI in the deciduous dentition and presence of DGI in the permanent dentition was high in all types of OI.

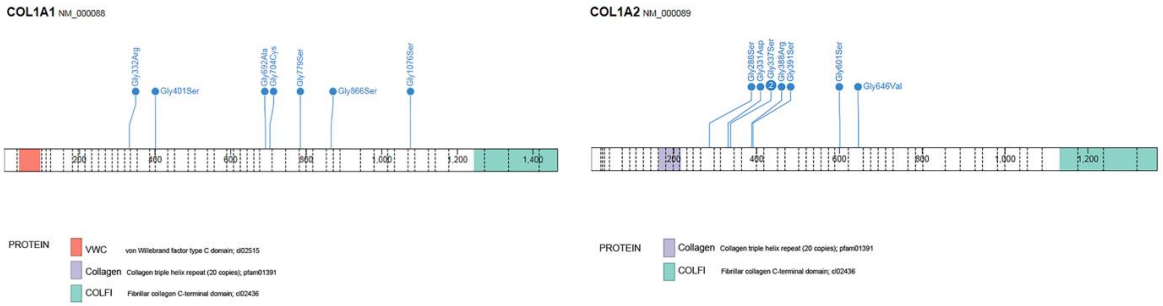

Figure 10. Location of mutations in *COL1A1* and *COL1A2* in children and adolescents with dentinogenesis imperfecta in both dentitions. Distribution of missense mutations from N- to C-terminal. Affected residues are numbered from translation initiation.

Presence of isolated histological DGI

In 54% of the individuals without clinical or radiographic signs of DGI, a diagnosis of DGI was confirmed histologically. The histological changes were milder compared to those of the patients with clinical and radiographic signs of DGI. No individual with isolated histological DGI in the deciduous dentition exhibited signs of DGI clinically or radiographically in the permanent dentition.

Taurodontism

Taurodontism occurred in 18% of individuals who could be evaluated for this condition. The difference in presence of taurodontism between individuals with quantitative (18%) and individuals with qualitative (13%) defects was not significant.

Retention of permanent second molars

Of the study cohort, 31% exhibited retention of permanent second molars with 16% of these presenting with a quantitative defect and exhibiting retention compared to 50% of those presenting with a qualitative defect (p=0.003). Maxillary mesioangular retention was the most common inclination, 63%. Retention was more common in individuals with OI type III (69%) compared to the individuals with OI type I (19%) (p=0.001). Significantly larger values were found for the SN to SpPm angle for patients with retention in the upper jaw (p=0.017) and for those with retention in both jaws (p=0.003) compared to individuals in the reference population. Significantly lower values were found for the ML to SpPm angle for patients with retention in the upper jaw (p=0.044). No significant difference between individuals with OI and the reference population was found in the SpPm angle and ML. Presence of DGI or Pamidronate treatment had no significant effect on retention of permanent second molars.

Other rare gene variants in children with OI, hypodontia, and oligodontia (IV)

We detected a novel homozygous nonsense variant in *CREB3L1*, p.Tyr428*, c.1284C>A in one boy previously diagnosed with OI type III. Our patient presented with oligodontia and severe malocclusion including a mandibular overjet, unilateral open bite and bimaxillary crowding. However, no DGI could be detected clinically or radiographically. In the other cases, *COL1A1* and *COL1A2* were the only two common genes among all cohort individuals which carried a mutation. However, we found rare variants with unknown significance in several other genes related to tooth development. No significant differences in distribution of variants between individuals with oligodontia and severe hypodontia could be seen.

Among the detected variants, a missense variant in *AXIN2* (rs200883019; p.Thr511Met, c.1532C>T), segregating with tooth agenesis in an autosomal dominant manner, was identified in the family of a boy with OI type IV and oligodontia.

GENERAL DISCUSSION

OI is a complex connective tissue disorder with a wide spectrum of symptoms. Oral manifestations are common, rendering a need for correct diagnosis and restorative, prosthodontic and surgical treatment. The expressivity of DGI varies and setting a differential diagnosis between DGI-I and DGI-II is challenging. In this thesis, the aim was to map the extent of DGI-II in Swedish children and adolescents and to test the feasibility of differentiating

it from DGI-I in clinical dental practice. Furthermore, we aimed to elucidate the broad spectrum of dental and craniofacial manifestations in children and adolescents with OI related to genetic findings. We found a lower prevalence of DGI-II compared to previous studies. Collagen type I mutations that were predicted to induce a qualitatively changed protein product were associated with a higher prevalence of dental aberrations and malocclusion.

Prevalence of DGI type II

Prevalence studies serve many purposes. They are powerful tools for evaluating the extent of a disease in a specific population; thus, they are important for society and health services in the planning and allocation of resources needed to manage the condition. Comparison of disease prevalence between different areas within and between countries is also often important. Mapping occurrence of the disorder allows study of changes in severity and frequency over time. This thesis found an estimated prevalence for nonsyndromic DGI-II of 0.0022% (95% CI, 0.0016–0.0029%) or 2.2 affected in 100,000 individuals. This is significantly lower compared to previous studies reporting a prevalence between 0.013–0.1% (Witkop, 1957, Gupta et al., 2011, Yassin, 2016, Cassia et al., 2017).

The differences in frequencies clearly indicate the variance of this rare disease. We found a regional variation. This is not surprising as DGI-II is a disease inherited in an autosomal dominant manner with a low frequency of *de novo* mutations. Thus, individuals with DGI-II have a 50% chance of passing on the mutant gene and the disorder, to each of their children. Since it is likely that several individuals from the same family reside in the same area, the prevalence of this rare disease in that county would be significantly elevated. The loss of genetic variation that occurs in populations established from a small number of individuals and with a low degree of migration is substantially higher than in more heterogeneous populations with higher migration; the risk of pathogenic variants is thus higher in the former. Previous studies have been based on patient cohorts from one regional center, and the group of examined individuals may therefore not be representative for the country as a whole (Gupta et al., 2011, Yassin, 2016, Cassia et al., 2017).

Methodological differences may also be important for variance. The diagnostic criteria are of utmost importance for correctly identifying individuals presenting with the disorder. It is always wise to clearly declare the diagnostic criteria used in order to simplify comparability with new investigations. In the present cohort, we observed a prevalence of hypodontia of 9%, which is only slightly higher than reported for the population in Nordic studies (Aasheim & Ogaard, 1993, Backman & Wahlin, 2001, Haavikko, 1971). Another finding in the present

study was tooth retention or impaction (third molars excluded) in 17%. Retention of permanent second molars is a frequent finding in OI (Lukinmaa et al., 1987b, Malmgren & Norgren, 2002), but was not found in any individuals in this cohort of individuals with DGI-II. This indicates that retention of permanent second molars is more likely the result of the aberrant craniofacial development in OI than due to the bulbous crowns present in DGI.

We evaluated several variables indicative for OI that can be used in clinical practice to differentiate individuals with DGI-I and DGI-II. Using a structured questionnaire and an examination protocol with guidelines, pediatric dentists interviewed and examined patients regarding aspects such as bruising, prolonged bleeding, spraining, fractures, hearing impairment, and a family history of osteoporosis and OI. The degree of joint hypermobility and hue of sclerae, the whites of the eyes, were also assessed. Based on these variables, we identified one individual, a 2-year-old boy with OI type IV whose diagnosis had been overlooked. Several individuals with DGI presented with one or a few of these symptoms. Referral for further medical evaluation of OI should be based on presence of several of these variables. It is reasonable to suggest that a history of fractures is the primary indicator that may raise suspicion. Clinical experience of assessing children with OI and DGI-I and DGI-II indicates that it is probably most relevant to first identify children with DGI and recommend further medical evaluation. The results show that it is possible to identify children with OI with this approach.

Tooth agenesis in OI

The frequency of hypodontia (11%) and oligodontia (6%) in the cohort of OI patients was high. Tooth agenesis has previously been reported in individuals with OI (Lukinmaa et al., 1987b, O'Connell & Marini, 1999). The Finnish study found 18% presenting with the trait compared with 10% in the O'Connell and Marini study.

Because we wanted to evaluate the spectrum of the genetic impact on tooth agenesis in OI, we decided to include only one child per family, in contrast to previous studies. Furthermore, we only assessed individuals over the age of 7 when all permanent tooth germs have reached a developmental stage enabling radiographic evaluation. The teeth most often congenitally missing were permanent premolars (91% of the missing tooth germs), indicating that agenesis in the premolar regions is a specific feature in individuals with OI. Only four lateral incisors were missing. Agenesis of permanent lateral incisors and lower second premolars are equally distributed in the general population (Brook, 1974, Chosack et al., 1975). Oligodontia was

more common in individuals with a mutation predicted to induce a qualitatively changed protein product, but as an individual factor, this mutation did not explain the phenotype.

We found an inter- and intrafamilial variability in the expression of tooth agenesis and DGI. The same variant in *COL1A1* (p.(Gly821Ser), c.2461G>A) was detected in two unrelated males with OI type IV. One of these presented with a *de novo* mutation, eight congenitally missing teeth, and no DGI. The other boy exhibited DGI, but no tooth agenesis. These findings led us to evaluate the potential of modifying genes that could explain the phenotypic differences.

We found that the boy with the variant in *COL1A1* (p.(Gly821Ser), c.2461G>A) and oligodontia also presented with a variant in *AXIN2* (p.Thr511Met, c.1532C>T). The proband's mother, grandfather, and an aunt, all carriers of the variant, were unaffected by OI but presented with hypodontia. Interestingly, none of them could be diagnosed with oligodontia. This finding indicates that the more severe phenotype seen in our proband may be due to additive effects of the variants in *COL1A1* and *AXIN2* and their modifying or interacting genes.

AXIN2 encodes the axis inhibition protein 2, a protein that facilitates β-catenin degradation by forming a destruction complex in the canonical Wnt signaling pathway. Wnt signaling plays a vital role in early tooth development (Chen et al., 2009) and is intense in the epithelial initiation centers and enamel knots (Liu et al., 2008, Ahtiainen et al., 2016). *AXIN2* may act as a Wnt feedback inhibitor. Several mutations in *AXIN2* have been associated with varying severities of hypodontia and oligodontia (Bergendal et al., 2011, Liu et al., 2015, Wong et al., 2014, Yue et al., 2016). The findings indicate that an increased Wnt/β-catenin signaling caused by altered function of *AXIN2* may cause tooth agenesis.

Our finding further indicates the possibility of parallel genetic events that may explain the expressed phenotype. Overlapping phenotypes may involve proteins that have close interaction at the molecular level or distantly at the level of the functional unit or organ system (Posey et al., 2017). Except for the variants we found in *COL1A1*, *COL1A2*, and *CREB3L1*, we were unable to identify any other mutual variant related to collagen type I that could explain the phenotype with OI and oligodontia. Based on our findings, we suggest that the cause of the expressed phenotype is the collagen I mutation, but that additive effects from rare variants in several other genes may be important.

Expressivity of DGI in relation to genetic findings in OI

The expressivity of DGI was varying and differed significantly between the deciduous and permanent dentitions. The deciduous teeth were always most severely affected. This finding is

in accordance with previous studies (O'Connell & Marini, 1999, Malmgren & Norgren, 2002). Presence of DGI in both dentitions was significantly more common in individuals with a mutation predicted to result in a qualitatively changed collagen type I. No significant difference between affected chains (α1 vs. α2) could be found. Phenotypic severity seemed to be gradient dependent. Substitutions of glycine residues for another amino acid in the triple helix were more severe when they occurred towards the carboxy-terminal end. We found that no individuals harboring mutations amino-terminal of p.Gly305 in *COL1A1* and p.Gly211 in *COL1A2* presented with DGI in both dentitions.

Our findings are in accordance with the gradient model proposed by Byers et al. (1991). Assembly of the three α chains initiates in the C-terminal portion of the molecule and proceeds towards the N-terminal end (Marini et al., 2017). The C-terminal substitution of glycine by another amino acid may cause prolonged retention of the procollagen chain in the ER, resulting in longer exposure to posttranslational modifying enzymes (Ishikawa & Bachinger, 2013). Possibly, this causes a greater portion of retained and degraded intracellular procollagen in the odontoblasts, and more severe disturbance of protein secretion due to odontoblastic dysfunction. It has been hypothesized that mutated procollagen chains could be intracellularly retained within the ER and within transition and secretion vesicles. This would cause the odontoblasts to become dysfunctional (Hall et al., 2002). Such a response by the odontoblasts may be in accordance with studies of the ER stress response of osteoblasts and fibroblasts, where the intracellular retention of abnormal collagen chains cause apoptosis activation, autophagy stimulation, and impaired osteoblast stimulation (Ishida & Nagata, 2009, Forlino et al., 2007, Bianchi et al., 2012, Gioia et al., 2012). A previous study found intracellular retention of mutant procollagen in fibroblasts that resulted in a reduced amount of collagen type I in the ECM (Bateman et al., 1984).

The next important step is the interaction between collagen type I and the ECM. When abnormal collagen is secreted into the matrix, the composition changes abruptly and interferes with mineralization. These abnormal chains assemble into abnormal fibrils, resulting in an aberrant ECM, indicating that the mutation plays a significant role in altering protein behavior on assembly. In study II, 54% of the patients without clinical or radiographic signs of DGI exhibited histologic findings of DGI. Interestingly, isolated DGI was more common in individuals with quantitative collagen I defects. The teeth exhibited less severe dentin manifestations. This finding indicates that the odontoblasts may also be affected in quantitative defects, but to a lesser extent. An interesting finding is that no individuals harboring a mutation causing a quantitatively changed protein exhibited any signs of DGI clinically or

radiographically. We thus suggest that presence of a qualitatively changed protein is mandatory for development of clinical and radiographic DGI.

The higher prevalence of DGI in the deciduous than the permanent dentition remains to be elucidated. The time needed for the odontogenesis of the deciduous compared to the permanent dentition is short. It is reasonable to propose that the genetic program regulating the odontoblastic activity is in specific ways variable and that the influence of epigenetic factors is important for the expressed difference. Initiation of odontoblastic differentiation starts at the future cusp tips. Several genes important for odontoblast differentiation have been found, including *WNT10A*. *WNT10A* is highly expressed in the mesenchyme inducing the preodontoblasts to become functional *DSPP*-expressing odontoblasts (Yamashiro et al., 2007). Other genes of importance are e.g. BMP/TGFβ. The expression of these genes during development may have different effects on the ability of odontoblasts to perform dentinogenesis in a space- and time-specific manner (Lesot et al., 2001).

Taurodontism

Taurodontism was a frequent finding in this cohort. The prevalence of 18% found was high compared to the prevalence in the general population (Backman & Wahlin, 2001, Gupta et al., 2011), but lower than in the Malmgren and Norgren study (Malmgren & Norgren, 2002), which reported a prevalence of 42%. We evaluated taurodontism only in mandibular permanent first molars, as this tooth was most reliably assessed based on radiographic projection. It is reasonable to expect that the frequency would have been higher if all permanent molars had been included. We found no correlation between taurodontism and any specific type of collagen I mutation (qualitatively or quantitatively changed protein). We hypothesize that our findings indicate the importance of collagen I as one of multiple components involved in the intricate epithelial-mesenchymal interactions in the morphologic development of permanent teeth. Understanding the mechanisms underlying these interactions remain to be elucidated. It has been suggested that the explanation to the characteristic morphology in taurodontism can be found in the HERS (Hamner et al., 1964). Root formation begins after the crown has been fully formed. During this process, the outer and inner dental epithelium fuse to form HERS. This process results in the differentiation start of the root odontoblasts. Taurodontism is a characteristic finding in tricho-dento-osseous syndrome (MIM 600525), caused by heterozygous mutations in the *DLX3* gene. *DLX3* is located on the same chromosome (17q21.33) and close to *COL1A1*. Furthermore, taurodontism has been observed in several other syndromes (Joseph, 2008, Axelsson, 2005, Islam et al., 2005, Jaspers, 1981). As HERS is induced before odontoblast differentiation, we suggest that it is more likely that earlier

aberrant collagen I expression in the mesenchyme may be important for epithelial-mesenchymal interactions during tooth morphogenesis. Furthermore, based on previous findings, taurodontism appears to be a polygenic trait, and the collagen I disturbance may have direct and indirect interactional effects on related genes and their protein products.

Retention of permanent second molars and craniofacial aberrations in OI

We found a high prevalence of retained permanent second molars (31%). Mesioangular maxillary retention was most frequent. Impaction of permanent second molars is an uncommon finding in healthy individuals (0–2.3%) (Bondemark & Tsiopa, 2007, Farman et al., 1978, Varpio & Wellfelt, 1988). Physical obstacles, crowding, and failures in the eruption mechanism have been proposed etiological factors for retention of permanent second molars (Magnusson & Kjellberg, 2009). We found that retention was more common in individuals harboring a qualitatively changed collagen type I. Furthermore, aberrant craniofacial development was seen significantly more often in individuals presenting with molar retention compared to individuals with OI and no retention in comparison with age- and gender-matched controls. It has previously been hypothesized that the retention of permanent second molars is caused by the bulbous crown of the permanent first molar (O'Connell & Marini, 1999). However, retention was also seen in individuals not affected by DGI. This finding is in line with Malmgren and Norgren (2002). Based on our findings, we suggest that the primary etiology in OI is lack of space in the maxilla. Aberrant craniofacial development in OI with a retrognathic maxilla has been reported in previous studies (Jensen & Lund, 1997, Waltimo-Siren et al., 2005).

The highly complex nature of craniofacial morphogenesis suggests involvement of a substantial number of genes, but to date only a few loci have been identified. Development of the craniofacial complex is modulated by sophisticated and exactly timed gene expression mediated by complex signaling pathways (Carlson, 2015). It is not reasonable to suggest that the collagen type I mutation itself is enough to explain the expressed phenotype; genes do not act in a vacuum. Compared with in non-affected individuals, unlike responses to hormones, nutritional status, and biomechanical factors may affect facial morphogenesis (Williams & Slice, 2010). Nevertheless, mandibular prognathism, lateral open bite, crossbite, and retention of permanent second molars are frequent findings in OI. Thus, it is reasonable to suggest that collagen type I defects are important. The bones of the jaws form through intramembranous ossification. Increases in the size of these bones are mainly driven by surface apposition, where

sutures are found (Waltimo-Siren et al., 2005). Based on our findings, it can be speculated that the defect collagen type I results in a direct or indirect structural and physiological change at these locations.

Methodological considerations

This thesis has strengths and limitations. The prevalence of DGI-II and the medical variables associated with OI were evaluated. One strength is that we searched for cases in all Swedish counties. The prevalence calculations were based on official statistics from the National Board of Health and Welfare. We assessed these data as reliable because we assumed a coverage ratio of > 95%. A limitation is that not all individuals born during the years 1996–2015 were examined regarding DGI-II. Obviously, this would be an impossible task. However, all Swedish children and adolescents have regular dental examinations. It is less likely that individuals with DGI-II are undiagnosed because of the severe expression of the disease. Furthermore, it can also be assumed that the majority of children with DGI-II have been assessed at specialist pediatric dental clinics. Attempts were made to contact clinics that did not report cases for several years. Other possible limitations include the presence of several practitioners, which reduces interobserver reliability.

The large cohort of children and adolescents with OI is the major strength of this study. New knowledge of rare diseases is mainly based on case reports. The possibility to retrospectively evaluate dental development of 152 children and adolescents with OI is a unique opportunity for increasing understanding of the distribution of dental and craniofacial disturbances in OI that clinicians treating these individuals should be aware of. Nevertheless, the retrospective design has limitations. One limitation is that observations were based on available data. This is clear due to the missing data for some variables. Prospective cohort studies provide a clear protocol for which variables should be documented and in what way.

This is the first time whole-genome sequencing was used to specifically describe the dental phenotype in OI. The approach resulted in detection of a rare homozygous variant in *CREB3L1* in one child and several rare variants of uncertain clinical significance in genes related to tooth development. The approach opens up the possibility of further evaluating the effects of these genes. The difficulty of interpreting data from whole-genome sequencing and small sample size are other limitations. WGS provides us with a wealth of information that needs to be properly assessed. We must be able to discriminate potentially pathogenic variants from benign variants that explain normal human heterogeneity. We used a large number of databases and prediction algorithms to assess the detected variants regarding expected severity. However, one

limitation is that we did not confirm results of all detected variants or examined relatives via Sanger sequencing. Such an approach would further assist in the filtering process. Furthermore, functional validation would be important to assess the effect of the variant on the protein function level.

Ethical considerations

Several ethical questions should be considered. The handling of genetic information is a responsibility. Detection of a pathogenic variant in a proband means that relatives may be carriers and prospective children may inherit the variant. Nevertheless, genetic testing is of utmost importance when diagnosing rare diseases. A rare disease is a health condition that affects a small number of individuals compared with other prevalent diseases in the general population (Richter et al., 2015). OI is by definition a rare disease. The interpretation of genetic data is challenging. To ascertain that this could be done in an appropriate way, clinical geneticists and a bioinformatician were involved to ascertain test accuracy and interpretation of results, and to handle potential post-testing complications. Several factors are relevant to consider in the decision making of genetic tests; these include integrity, beneficence, non-maleficence, and justice. However, several additional variables should be considered. Dilemmas arise when any of these variables cannot be satisfied. We need to ask ourselves: Who will obtain positive effects of the test? Is there anyone who may be harmed by the information? All factors need to be weighed in order to make an assessment. If the patient consents to testing, we must consider the individual's right to know, but also the right not to know. Feedback of the obtained information must be handled in a reflective way. Results revealing a clinically relevant condition that can be treated during childhood should be reported to the legal guardians. The identification of pathogenic variants in *COL1A1* and *COL1A2*, which cause OI, may be such an example. There is a great diagnostic value for the patient and clinician, where the benefits outweigh the risks as a diagnosis gives the opportunity to treat all aspects of the disease.

Increasing our understanding of the full spectrum of symptoms by assessing many individuals with a rare disease is also important. Diagnostic delay is frequent in individuals with rare diseases. By assessing more individuals, it is possible to increase the understanding of the breadth of the symptoms. In the long run, this knowledge may increase the ability to correctly diagnose children with the disorder and identify medical and dental problems at an earlier stage. Because of the impact on the child's physical and mental health, it is of value for the individual to obtain a correct diagnosis as early as possible. As we increase our understanding of the human genome and the variants seen in rare diseases, better and more sensitive diagnosis and

treatment become possible. It is our hope that these pieces in the jigsaw puzzle will be of value for our future ability to personalize medicine for treatment, and a potential cure.

MAIN FINDINGS AND CONCLUSIONS

Prevalence of DGI-II in Swedish children and adolescents

This thesis found a DGI-II prevalence of 0.0022% or 2.2 in 100,000 children and adolescents. Among the reported cases of DGI-II, we identified one child with undiagnosed OI.

Tooth agenesis in OI related to mutations in the collagen type I genes and genes related to tooth development

A high prevalence of tooth agenesis was seen in children and adolescents with OI. Collagen I mutations are associated with congenitally missing teeth and significantly more frequently seen in individuals with DGI, and in OI type III compared to type I or IV. Oligodontia is more common in individuals harboring collagen type I mutations predicted to cause a qualitative defective protein. We detected several rare variants of uncertain clinical significance, in genes associated with tooth development. Based on the findings in this thesis, we suggest that the cause of the expressed phenotype is the collagen I mutation, but that additive effects from rare variants in several other genes are important.

Mutations in *COL1A1* and *COL1A2* and dental aberrations in children and adolescents with OI

Our results indicate a higher risk of developing multiple dental aberrations including DGI, taurodontism, retention of permanent second molars, and craniofacial developmental disturbances in children and adolescents with OI. The presence of DGI, and retention of permanent second molars due to deviating craniofacial development is strongly associated with mutations predicted to cause a qualitatively changed protein. Collagen chain mutation position is correlated with the risk of developing DGI in both dentitions. Based on the findings in this thesis, taurodontism and retained permanent molars are clinical signs that support further clinical investigation to rule out a mild form of OI in an individual that is fracture prone. Finally, our results highlight the importance of carefully assessing and following children and adolescents with OI clinically, radiographically, and histologically over time in order to obtain correct diagnoses and identify potential oral complications at an earlier age. Genetic analysis can be helpful in identifying individuals with an increased risk of dental and craniofacial disturbances.

CLINICAL IMPLICATIONS AND CONSIDERATIONS IN OI

OI is a disorder associated with a range of oral complications due to the mutations in *COL1A1*, *COL1A2* and potentially genes involved in the transcription and posttranslational modification of type I collagen. The findings in this thesis highlight the complexity of the traits and the high inter- and intravariability in individuals harboring the different mutations.

Diagnosis of DGI-II and differential diagnosis of DGI-I

In this thesis we investigated the prevalence and incidence of DGI type II in Swedish children and adolescents. We further evaluated the presence of medical and dental variables associated with OI in these children. We detected one boy with previously undiagnosed OI. Our findings underscore the importance of evaluating OI symptoms in children and adolescents diagnosed with DGI-II. The protocol used in this study can be used for this purpose. The variables include questions regarding bruising, prolonged bleeding, spraining, fractures, hearing impairment, and family history of osteoporosis and OI. Assessment of joint hypermobility and sclerae may also be included in the examination. Clinical dental variables associated with OI include presence of retained permanent second molars, malocclusion, and DGI indicators – pathologic discoloration, attrition, and fractures. Radiographically, number of tooth germs, extended pulp chambers (taurodontism), and DGI indicators such as bulbous crowns with cervical constriction, pulpal obliteration, and short roots may be evaluated. Heredity of DGI is also an important indicator to consider for differential diagnosis as *de novo* mutations in the *DSPP* gene are uncommon. Our findings also indicate that DGI type II is less common compared to OI (2.2 per 100,0000 individuals compared to 7.4 per 100,000 individuals) (Lindahl et al., 2015).

Tooth agenesis

OI is associated with an increased risk of hypodontia as well as oligodontia. Based on our findings, it is important to diagnose agenesis at an early stage. We suggest that a panoramic radiograph be made at 7 years of age in children and adolescents with OI. We detected oligodontia in all types of OI. This finding indicates that severe agenesis may also be found in an individual presenting with OI of mild severity. Furthermore, our findings indicate that the possibility of undiagnosed OI should be considered in individuals presenting with oligodontia and hypodontia. Further evaluation regarding presence of the above mentioned medical and dental variables associated with OI may be indicated in such an evaluation. If suspicion of OI remains, referral to a pediatrician is indicated. Highly specialized competence is available at

the Swedish national multidisciplinary pediatric OI team at Astrid Lindgren Children's Hospital at Karolinska University Hospital for evaluation.

Diagnosis of DGI-I

We found that risk of developing DGI in both dentitions differed depending on the localization of mutations in the collagen type I chains. Our findings can be useful in clinical consultations to better answer questions from parents regarding whether their child presenting with DGI in the deciduous dentition also has an increased risk of an affected permanent dentition. Children with no DGI in the deciduous dentition and a mutation located on the N-terminal of p.Gly305 in *COL1A1* and p.Gly211 in *COL1A2* did not develop DGI in the permanent dentition. The deciduous dentition is always the most severely affected. Those presenting with only subtle histological signs of DGI in the deciduous dentition did not develop clinical or radiographic signs of DGI in the permanent dentition. The high frequency of mutations that induce quantitative defects stress the importance of histologic analysis of exfoliated teeth also in mildly affected children and adolescents with OI.

Taurodontism

Taurodontism is a frequent finding in OI. Our findings indicate the importance of collagen type I as one of several components involved in the intricate epithelial-mesenchymal interactions in the morphologic development of permanent teeth. Children with OI and taurodontism only occasionally exhibited the more severe form of taurodontism. Subjective complaints are rare; however, presence of taurodontism can be challenging in cases that require endodontic treatment. The complicated variation of canal configuration and apical displacement of the orifices stress the importance of magnification for successful instrumentation and obturation.

Retention of permanent second molars

We found a high prevalence of permanent second molar retention. Most were located in the maxilla. The presence of one or multiple retained permanent second molars was significantly associated with mutations in *COL1A1* or *COL1A2* predicted to cause qualitatively changed collagen type I. Thus, special emphasis should be put on diagnosing retention in children harboring this class of mutations. Early diagnosis is important in order to direct development of the occlusion positively and prevent periodontal problems, follicular cysts, pericoronal inflammation, and pain. Failure to detect retention may occasionally cause root resorption of the permanent first molar. Our clinical experience is that surgical exposure may facilitate eruption when retention is diagnosed and no other obstacle (e.g. impacted third molars) is

present. Our clinical practice is to pause bisphosphonate treatment 2 weeks before and 6 weeks after the surgical procedure in order to minimize the risk of bisphosphonate-related necrosis of the jaw. However, an individual assessment must always be done. It is wise to contact the Swedish national multidisciplinary pediatric OI team at Astrid Lindgren Children's Hospital before the surgical procedure.

General management of individuals with OI clinically in dental practice

The dental management of children and adolescents with OI also include other important variables. Bleeding and easy bruising is a common feature in heritable connective tissue disorders, including OI. The coagulation defect is partly related to the effect of abnormal collagen on platelet–endothelial cell interactions and capillary strength (Keegan et al., 2002, Malfait & De Paepe, 2009, Siegel et al., 1957). There is a 10-30% frequency of bleeding diathesis in individuals with OI, and the risk of bleeding complications when performing oral surgery procedures in these children is increased. Thus, it is wise to discuss the case with medical colleagues before electing surgical procedures in order to set in prophylaxis or manage bleeding complications.

Craniofacial and dental aberrations are common in individuals with OI. Class III malocclusion, lateral open bite, crossbite and agenesis are frequently seen. Our experience is that a restrictive approach should be taken to early interceptive treatment. Later signs of aggravating malocclusions might occur. When facial growth and dental development are finished orthodontic treatment with fixed appliance and/or orthognathic surgery might be necessary.

The potential presence of atlantoaxial instability in some individuals with OI must always be considered. It is characterized by an excess mobility of atlas (C1) and axis (C2) due to the manifestation of collagen type I defect in the surrounding ligaments. Before tipping patients with OI backward in the dental chair, it is important to be aware of potential involvement of this feature and be careful during operative procedures as an incautious handling may result in severe spinal cord complications. It may be wise to let the parent lift a child with high fracture risk to the operating table.

Malignant hyperthermia is uncommon but can occur during anesthesia.

FUTURE RESEARCH

The results of these studies indicate the complexity of the human genome and its resulting phenotype. It is obvious that collagen type I mutations do not act in a vacuum. One significant variable to be aware of is the difference between the actual DNA sequence change, and the resulting protein product and its interactions. The phenotype resulting from the collagen type I mutation is an obvious example. It points to the importance of further elucidating potential mechanisms underlying the expressed phenotype. Based on the results of these studies, it would be desirable to further evaluate the genetic programs governing tooth and craniofacial development in OI in animal models.

One limitation of our studies is that we only assessed nucleotide changes at the DNA level. It would be desirable to further evaluate changes at gene expression level by verifying functional analysis. A natural continuation of the present results would be to test the effects of specific mutations in, for example, animal models. This is also a challenging task. Loss-of-function mutations would be the easiest way to test by knockout of specific genes of interest. Several animal models have been used in the investigation of OI, fewer have been developed to further investigate the dental phenotype. These include the Mov-13 mouse (OI type I), Brittle II mouse (OI type II), Oim/Oim mouse (OI type III), and Brittle IV mouse (OI type IV).

Zebrafish would be a potentially interesting animal model to further develop and evaluate. Short generation time, reliable mutagenesis techniques, and a huge number of embryos are some of the advantages of a zebrafish model for skeletal dysplasias (Fisher et al., 2003). Heterozygous Chihuahua fish express phenotypic similarity to humans and could be a promising model to use for further investigation of tooth and craniofacial development and the effects of specific gene alterations. Regarding *COL1A1*, the DNA sequence is highly similar to the human sequence. A 76% similarity exists at the amino acid level (Kamoun-Goldrat & Le Merrer, 2007). The Chihuahua mutants could be a model for further assessment of tooth and craniofacial development in OI based on both molecular defects and the resulting phenotype, including misshapen and irregularly shaped bones. Zebrafish homolog models have been used to assess several syndromes in recent years; this includes the *sec24d* model for OI (Sarmah et al., 2010).

We identified several variants of uncertain clinical significance in genes with a known impact on tooth development. We critically revised all these variants based on several algorithms to assess their potential impact on phenotype. However, in order to be more certain about the pathogenicity, functional validation studies are necessary. Here, animal models may be helpful. One advantage of animal models is that this approach results in the specific genetic variant

effects on the whole organism. Knockouts of specific genes could be used to study the influence on other potentially interacting genes. Another interesting aspect could be to study the expression of developmental genes at specific locations during the various stages of craniofacial and tooth development. This could be done by, for example, tagged antibodies in western-blotting or enzyme-linked immunosorbent assay (ELISA). It is intriguing to speculate that these kinds of studies could give us more clues about the time- and space-specific alterations and interactions during tooth and craniofacial development. In future, more knowledge on these genetic alterations may give us better opportunities to guide development of the individual in the most favorable way during treatment. In the long run, it is intriguing to consider the development of gene therapeutic methods to treat the conditions associated with the disease.

To enable future gene therapeutic alternatives, we need to increase our understanding of the underlying genetic changes and its effects. These kinds of studies stress the importance of large cohorts of individuals presenting with the rare disease. A further perspective on the first study in this thesis would thus be further genetic evaluation of the included individuals. The identification of families in whom DGI-II is present opens up the opportunity for later investigations of genotype-phenotype correlations. Such evaluation could provide a unique opportunity to further elucidate genetic similarities and differences and the expression of the disease in an extensive cohort of this rare disease. The focus of such genetic evaluation could reasonably be the *DSPP* gene.

Finally, considering the results of the fourth study in this thesis, we encourage the use of next-generation sequencing to identify disease-causing variants in OI and other rare diseases. However, to identify potential common variants in individuals presenting with a specific rare phenotype, it would be valuable to include more individuals and controls to enable discrimination of potential gene candidates. Furthermore, in addition to our *in silico* predictions, investigation of relatives and confirming functional validation is essential for identifying true disease-causing variants.

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